Differential Binding of Traffic-related Proteins to Phosphatidic Acid- or Phosphatidylinositol (4,5)-Bisphosphate-coupled Affinity Reagents*

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Phosphatidic acid (PA) is an important bioactive lipid, but its molecular targets remain unknown. To identify such targets, we have synthesized and coupled PA to an agarose-based matrix, Affi-Gel 10. Using this matrix as an affinity reagent, we have identified a substantial number of potential PA-binding proteins from brain cytosol. One class of such proteins is known to be involved in intracellular traffic and it included coatomer, ADP-ribosylation factor (Arf), N-ethylmaleimide-sensitive factor (NSF), and kinesin. Binding of these proteins to PA beads was suppressed by soluble PA, and it occurred preferentially over binding to beads coupled to phosphatidylinositol (4,5)-bisphosphate. For coatomer, Arf, and NSF, we verified direct binding to PA beads using purified proteins. For recombinant Arf1 and Arf6, binding to PA required myristoylation. In addition, for NSF and Arf6, an ATPase and a GTPase, respectively, binding to PA beads was extremely sensitive to the nucleotide state of the protein. Binding to PA may be a property linking together distinct participants in one complete round of membrane transport from a donor to an acceptor compartment.

The lipid membranes of eukaryotic cells are dynamic in composition and appear to be ideal nucleation sites for selective recruiting in a time-dependent manner a variety of cytosolic proteins to specific microdomains. Diacylglycerol was among the first lipids to emerge as a specific binding partner for protein kinase C, and more recently the phosphoinositides are attracting considerable attention in their roles of recruiting important signaling proteins to defined membrane compartments (1–5).

PA synthesized via the glycerol-3-phosphate or the hydroxyacetone phosphate pathway is an important intermediate in the biosynthesis of glycerolipids and triacylglycerols (6). The contribution of PA to lipid-based signaling is less well understood. Apart from the biosynthetic routes mentioned above, PA can be rapidly elevated in cells through hydrolysis of phosphatidylcholine by phospholipase D (PLD) (7, 8). Because activation of PLD has been implicated in the regulation of cellular processes ranging from growth control to traffic, it is generally assumed that PA-interacting proteins downstream of PLD activation exist to mediate those functions (9, 10). However, because PA elevated through PLD activation is unstable and rapidly converted to diacylglycerol via hydrolysis by PA hydrolases, in pathways where PLD activation is measurable it is not always clear whether PA or diacylglycerol is the relevant signaling lipid (11).

One protein that has been shown to interact directly with PA is the serine/threonine kinase Raf-1, a component of the mitogen-activated protein kinase cascade (12, 13). Translocation of Raf-1 from the cytosol to intracellular membranes (primarily endosomes) depends on the presence of PA and can be blocked by point mutations that abolish this interaction. The discovery of at least one protein that appears to interact directly with PA raises the possibility that additional such proteins exist, and their identification should be of considerable interest.

In addition to a function in signaling, PA has been proposed to have a role in intracellular traffic. It was originally shown that artificial vesicles made with a mixture of lipids including PA were better able to bind coatomer (a coat protein complex involved in transport between the endoplasmic reticulum and the Golgi complex) than their counterparts prepared without PA (14). In addition, assays that recapitulate vesicle formation in vitro with pure lipid and protein components revealed that acidic phospholipids such as PA enhanced vesicle formation (15–17). In other experiments, PA supplied exogenously to cells was able to rescue a block in endoplasmic reticulum to Golgi transport of viral proteins imposed by primary alcohols (18). In addition, altering PA levels was shown to disrupt the structure of the Golgi complex and affect traffic through this organelle (19). Recent reports have also indicated that PA synthesized via acylation of lysoPA may be involved in vesicle fission ("pinching off") in the Golgi complex or at the plasma membrane (20, 21). The inherent instability of PA as discussed above complicates any attempt to assign significance to PA as opposed to diacylglycerol in some of these experimental settings. More importantly perhaps, because PA can stimulate formation of PI (4,5)P2 by phosphatidylinositol 4-phosphate 5-kinase directly (22, 23), the search for the relevant lipid(s) in those pathways becomes even more complex and must include PI (4,5)P2. PI (4,5)P2 has been shown to influence on its own the activation cycle of several small GTPases such as Arf that are...
crucially involved in membrane transport pathways (24). PI (4,5)P_2 also interacts with other proteins involved in trafficking such as dynamin and clathrin-associated protein complex 2 (25).

One way to identify PA-binding proteins is to use this lipid in an immobilized form and to isolate proteins that bind to it. A similar reagent containing immobilized PI (4,5)P_2 can be used in parallel to determine whether candidate proteins show differential affinity for those two lipids. In this work, we have synthesized and used such reagents to identify a set of trafficking-related proteins that showed strong binding to immobilized PA but not to immobilized PI (4,5)P_2.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Affi-Gel 10 PA and Affi-Gel 10 PI (4,5)P_2 Matrices—**The amino functionalized PA (Fig. 1A, 2) was prepared in seven steps from the commercially available 5(S)/5(-)-1,2,3,4-tetra-O-isopropylidenglycerol (Fig. 1A, 3) (Aldrich). The coupling of 2 with the N-hydroxysuccinimide-activated ester-agarose resin, Affi-Gel 10 (Bio-Rad), required the solvent combination chloroform-water (0.8:1.0:0.2) to afford the PA-affinity reagent (1). Excess resin (~5 equivalents of N-hydroxysuccinimide groups) was used. The resulting loading capacity was estimated to be 2.6 μmol/ml. The estimation was done by 1H NMR analysis of the concentration of residual 2 in the reaction mixture in the presence of the internal standard 1,3,5-nitro-2-isotoluephosphate. In addition to the 2-palmitoyl derivative shown here, a 2-lauroyl derivative was prepared that showed identical binding characteristics. As a control of binding specificity we have also prepared and employed a 3-(β-aminoacyl)-functionalized PI (4,5)P_2 (Fig. 1B, 4) coupled via the amino function to an Affi-Gel 10 solid support. Details of both syntheses will be reported separately. 2

**Preparation of Membrane and Cytosolic Fractions from Sheep Brain—**All steps including centrifugation were carried out at 4 °C. 180 g of deep-frozen sheep brain was broken into small pieces and resuspended in ice-cold buffer (50 mM Tris-HCl, pH 7.6, 80 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of antipain, pepstatin A, aprotinin, and leupeptin) to a volume of 500 ml for 10–15 min until thawed. The tissue was then blended in a polytron until it reached a fine consistency and was centrifuged at 10,000 rpm for 30 min. The supernatant from this spin contained the membrane and cytosolic fractions. For preparing cytosol, the supernatant from above was centrifuged again for 1 h at 100,000 × g. The new supernatant was dialyzed for 24 h against buffer containing, pH 7.2, and 1% Nonidet P-40, 0.6 mM phenylmethysulfonyl fluoride, and 1 μg/ml aprotinin, and 1 μg/ml trypsin inhibitor on ice and centrifuged at 70 °C. The pellet after this spin (membranes) was resuspended in homogenization buffer (20 mM Hepes, pH 7.2, 1 mM EDTA, 0.2 μM sucrose plus protease inhibitors as above) and placed in a steel Dounce homogenizer for 20 strokes. The homogenate was centrifuged at 2500 rpm to remove debris, and the supernatant that contained membranes was centrifuged at 100,000 × g for 1 h. The pellet after this spin (membranes) was resuspended by homogenization to a total of 20 ml of homogenization buffer, frozen in liquid nitrogen, and stored at −70 °C.

**Binding Reactions and Regeneration of Beads—**Cytosol at 6 mg/ml was mixed 1:1 with lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM EDTA, 1% Nonidet P-40, 0.6 mM phenylmethysulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml trypsin inhibitor) on ice and centrifuged at maximum speed in a microcentrifuge to remove any aggregates. The PA beads, which were stored in water plus 0.02% sodium azide, were equilibrated with three washes in ipp buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Tween 20, and 0.02% sodium azide) and were resuspended in this buffer to 10% (w/v) unless otherwise stated. In a typical binding reaction, 450 μl of diluted cytosol were mixed with 35 μl of bead solution in a 500-μl microcentrifuge tube on ice. A few air bubbles were created to effect better mixing. The tubes were put in a rotator at 4 °C for 1.5–2 h. Following binding, the beads were washed quickly three times with ipp buffer and resuspended in Laemmli sample buffer, and bound proteins were analyzed by SDS-PAGE. In some experiments, solvent PA was included in the binding reaction as follows: L-α-phosphatidic acid (diacetyl, dilauroyl, or dipalmityl purchased from Sigma) that was dissolved in chloroform in 10 mg/ml and kept at −20 °C was warmed to room temperature and put into a glass tube. The lipid solution was dried for 10 min under a stream of nitrogen. To the dried lipid film, lysis buffer was added carefully to avoid creating any bubbles. The tubes were then sonicated for 5 min until a clear solution was obtained. This solution was stable for a few hours at room temperature. After resolubilizing, the beads were ready to use. In competition experiments, the cytosol was mixed 1:1 with lysis buffer that had been supplemented with PA solution and kept on ice for 15 min. Lipid beads were then added, and the binding reaction was carried out as described above.

The beads could be reused up to five times. At the end of an experiment, beads were collected using ipp buffer and centrifuged. To the pellet, a single buffer (50 mM Tris-HCl, pH 7.4, 2% SDS, 100 mM dithiothreitol) was added to make a 25% (v/v) suspension (54), and the suspension was allowed to proceed at 37 °C, for 2 h. The digest supernatant was acidified with 2 μl of 10% formic acid, and peptides were recovered by immobilization and subsequent desalting on a Vydac C8 guard cartridge (μPurifier™; LC Packings, Amsterdam, The Netherlands). Washing was with 1% formic acid, and peptides were eluted to a nanospray needle (Protana, Odense, Denmark) using 1–2 μl of 0.1% formic acid, 70% methanol. Peptide masses were examined using a Finnigan LCQ ion trap mass spectrometer fitted with a Protana nanospray interface. All significant peaks were examined for charge state and monoisotopic mass value in zoomscan mode and subjected to collision induced dissociation. Mass/fragmentation data sets were submitted to Mascot for data base searching (55). Results were scored by Mascot, and only those scoring significantly higher (p < 0.05) than a chance match were regarded as positive identifications. Only peptides giving matching fragmentation data were scored; matching by peptide mass only was not included. The number of peptides matched per protein discussed in this work were 12 for kinesin, 17 for proline-rich sensitive aminopeptidase, 12 for neurochondrin, and 5 for NSF. Antisera and Purified Proteins—The following antibodies were used in immunoblots: mouse monoclonal 1D9 to Arf, a kind gift of Dr. Richard Kuhn; mouse monoclonal M3A5 to β-cop, a kind gift of Dr. Thomas Kreis; mouse monoclonals H1 and H2 to brain kinesin, a kind gift of Dr. George Bloom; mouse monoclonal to PLC6, a kind gift of Drs. Matilda Katan and Matthew Jones; rabbit polyclonal to protein kinase C, a kind gift of Dr. Null Divecha; mouse monoclonal 100/2 to α-adaptin (AP-2 coat) and 100/2 to γ-adaptin (AP-1 coat) purchased from Sigma; and mouse monoclonal 6B7–3 to α/β-SNAP and 9G7–3 to NSF purchased from Stressgen. The following purified proteins were used: native coatamer, a kind gift of Dr. Gerry Waters, 50% pure, prepared and used as described (14, 37); native Arf, 20% pure and containing primarily (>70%) Arf1, a kind gift of Dr. Alex Brown, prepared and used as described (56); and recombinant NSF and SNAP, more than 90% pure (46), a kind gift of Dr. Sidney Whiteheart.

**RESULTS**

**Synthesis of PA and PI (4,5)P_2 Matrices—**To identify potential PA-binding proteins we synthesized a solid phase PA-based matrix as shown in Fig. 1A. In this reagent, the head group of the phospholipid is expected to be exposed to the solvent (and therefore available for binding), whereas the fatty acid at the 3 position anchors the PA to the Affi-Gel 10 resin. Although the 2-palmitoyl derivative is shown here, we have also synthesized and used a 2-lauroyl derivative, which showed identical binding characteristics. In addition, we have used a 2-palmitoyl PI

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(4,5)P₂ immobilized Affi-Gel 10 reagent (shown in Fig. 1B) to directly compare affinities of candidate proteins to the two lipids.

Binding of Cytosolic Proteins to PA Beads—We initially used brain cytosol as a source of material. In preliminary experiments we found that cytosol without detergent gave unacceptably high binding to the beads (data not shown). We used nonionic detergent in the cytosol solution to overcome this problem, because other types of detergent (ionic or zwitterionic) appeared to reduce binding to very low levels. When cytosol supplemented with Nonidet P-40 was incubated with the PA beads and unbound material was removed with several washes, a reasonable number of polypeptides were seen to be in the PA-bound fraction (Fig. 2). This pattern of staining is very reproducible. If those polypeptides were binding specifically to immobilized PA, then soluble PA should reduce their binding to the beads. Initial attempts at such inhibition of binding using dipalmitoyl PA (the same lipid as that coupled to the beads) were unsuccessful, perhaps because of the poor solubility of this lipid. Inhibition of binding was obtained, however, with shorter chain analogs of PA (dilauroyl, C:12 or dioctanoyl, C:8), which also showed superior solubility and sonication properties. When cytosol was preincubated with soluble dilauroyl PA, a substantial number of PA-bound bands were reduced in intensity (Fig. 2, asterisks). Interestingly, the presence of soluble PA also seemed to enhance the binding of some proteins to the PA beads, whereas binding for other polypeptides was unaffected. Here we deal with polypeptides whose binding to the PA beads is reduced in the presence of soluble PA.

Mass spectrometry was used to establish the identity of the PA-binding polypeptides. In addition, we determined directly the identity of some polypeptides using immunoblotting. From both protocols, we have identified 15 known and 5 novel proteins in the PA-bound fraction from brain cytosol. An additional 8–10 polypeptides have been obtained from brain membranes extracted with detergent, and their identification by mass spectrometry is in progress. In this work, we will discuss proteins involved in intracellular traffic (coatomer, Arf, kinesin, and NSF) whose binding to PA beads has been characterized further. From the remaining known proteins, we have cloned, expressed, and verified binding to PA beads of nordin (or neurochondrin), thought to be involved in somatodendritic functions in neuronal cells (26, 27), and puromycin-sensitive aminopeptidase, a protease thought to be involved in cell growth (28). The Raf-1 kinase bound to the PA beads at levels comparable with neurochondrin and puromycin-sensitive aminopeptidase.

Identification of β-cop Coatomer, Arf, NSF, and Kinesin in the PA-bound Fraction—Immunoblotting of PA-bound fractions with antibodies to the β-cop coatomer subunit or to native Arf revealed binding of these proteins to the PA beads (Fig. 3A). There was no binding to uncoupled Affi-Gel beads or aggregation in the absence of any beads (data not shown). More importantly, β-cop and Arf bound weakly to 2-palmitoyl PI (4,5)P₂ beads (Fig. 3A), indicating that binding was to the head group and not to the acyl chain of PA. To verify that the PI (4,5)P₂ beads bound proteins known to interact with this lipid, we
was undetectable PLC by washes, SDS-PAGE, and electrotransfer. The blots were probed with bind to the PA or PI (4,5)P2 beads (Fig. 3 serine) and to diacylglycerol (a dephosphorylated PA) did not additional control of the specificity of binding, we determined bind PI (4,5)P2 through its pleckstrin homology domain with a brain cytosol was mixed with PA beads as shown for 1 h. The concentration of lipid on the input beads was 150 nmol for PA and 120 nmol for the 2× PI (4,5)P2. Following washes, the proteins bound to the beads were analyzed by SDS-PAGE and antibodies to β-cop, PLCβ, Arf, or protein kinase C (PKC). B and C, brain cytosol was mixed with PA beads for 1 h followed by washes, SDS-PAGE, and electrotransfer. The blots were probed with antibodies to NSF or to α/β SNAP (B) or with two anti-kinase antibodies (C). D, soluble dilauroyl PA was added to the binding reaction at the concentrations shown for 15 min before the addition of PA beads. Following binding, SDS-PAGE, and electroblotting, the blots were probed with antibodies to β-cop, Arf, and kinesin as shown.

probed the blots with antibodies to PLCβ1, a protein known to bind PI (4,5)P2 through its pleckstrin homology domain with a $K_d$ value in the range of 0.1–10 μM (29, 30). Under conditions where binding of β-cop and Arf to PI (4,5)P2 was minimal, there was strong binding of PLCβ (Fig. 3A). Conversely, there was undetectable PLCβ bound to the PA beads (Fig. 3A). As an additional control of the specificity of binding, we determined that protein kinase C, a protein abundant in the cytosol and known to bind to acidic phospholipids (such as phosphatidylinerine) and to diacylglycerol (a dephosphorylated PA) did not bind to the PA or PI (4,5)P2 beads (Fig. 3A).

Additional traffic-related proteins were identified from mass spectrometric analysis of the PA-bound bands shown in Fig. 2. One such was NSF, an ATPase involved in numerous transport steps (31, 32), and its presence in the PA-bound fraction was verified by immunohblotting (Fig. 3B). Interestingly, little SNAP (soluble NSF attachment protein), which is a binding partner of NSF (33), was detected in the PA-bound fraction (Fig. 3B). A second protein identified from mass spectrometric analysis of the PA-bound bands shown in Fig. 2 was the heavy chain of kinesin, a microtubule motor protein involved in numerous transport steps (34, 35). The presence of kinesin in the PA-bound fraction was verified using two monoclonal antibodies to this protein (Fig. 3C) (36). There was no NSF or kinesin bound to uncoupled or to PI (4,5)P2 beads (data not shown but see Fig. 3). For all traffic-related proteins discussed above, we verified that binding to PA beads could be competed with exogenously supplied short chain PA (Fig. 3D and data not shown for NSF). In all cases, dilauroyl PA inhibited binding at lower doses than dioctanoyl PA (data not shown). We speculate that this may be related to the height of the head group in the mixed micelles used for competition, with the “taller” dilauroyl lipid having a more accessible head group to serve as a competitor in the binding reaction. Because we used the shorter chain lipid in the competition reaction, we also synthesized the 2-lauroyl-PA resin and were able to show that its binding characteristics were very similar to the 2-palmitoyl counterpart (data not shown). We have tried to compete binding to PA beads with a number of other reagents, in all cases obtaining negative results. Those included phosphate ions (up to 100 mM), glycerol-phosphate (up to 50 mM), ATP (up to 10 mM), GTP (up to 10 mM), and dipalmitoyl phosphatidylcholine (up to 300 μM) (data not shown).

Binding of Coatomer Complex but Not of Other Coats to PA Beads—The observed binding of β-cop to PA beads raises the question of whether the entire coatomer complex (37) is present in the PA-bound fraction and whether binding is direct. Using native coatomer that was 50% pure and did not contain any Arf, we found all coatomer subunits in the PA-bound fraction (Fig. 4A, lanes 6 and 7). Prominent noncoatomer-related bands contaminating the starting preparation were totally excluded from the bound fraction (Fig. 4A, bands marked with asterisks). We also attempted to disassemble coatomer using high salt to find which of the subunit(s) contain(s) PA-binding sites. We were unable to answer this question because conditions of disassembly also interfere with binding to the PA beads (data not shown). It was of interest to ask whether coats related to coatomer also showed binding to PA beads. The PA-bound fraction from cytosol was resolved in triplicate and probed with antibodies to β-cop, γ-adaptin (a subunit of the AP-1 coat involved in Golgi traffic (38)) and α-adaptin (a subunit of the AP-2 coat involved in endosome and plasma membrane traffic (39)). Only β-cop showed strong binding to the PA beads (Fig. 4B).

Because coatomer and Arf are capable of interacting in vivo (40, 41) and in vitro (42, 43), we investigated whether the presence of one would affect the ability of the other to bind to PA beads under nonactivating conditions for Arf. When purified coatomer was incubated with PA beads in the presence of increasing amounts of purified Arf, there was no effect of Arf on the amount of coatomer bound (Fig. 4C). The converse experiment (increasing coatomer in the presence of constant Arf) showed similar results (data not shown). Thus, both coatomer and Arf bound to PA independently of each other and without assistance from other factors. For interaction of activated Arf with coatomer during PA binding, see below.

Arf Binding to PA Beads Requires Myristoylation and Depends on Nucleotide State—We adapted the binding reaction to lysates from tissue culture cells, because this would allow us to address binding of candidate proteins after transient overexpression or after manipulating cellular function. We found that lysis with nonionic detergent (0.4% Nonidet P-40) but not with other types of detergent maintained the specificity of binding (data not shown). Using those conditions, we examined PA and PI (4,5)P2 binding of transiently expressed Arf1 and Arf6 mutants.

Most members of the Arf family are cytosolic proteins which translocate to membranes upon binding GTP (44). Binding to PA in this context may be understood as an additional way to enhance the affinity of the activated protein to membrane
subdomains. A divergent member of the Arf family is Arf6, which is membrane-bound and cycles between endosomal and plasma membranes depending on its activation state (45). It was therefore of interest to determine whether Arf6 also bound to PA beads. COS cells were transfected with plasmids encoding hemagglutinin-tagged Arf6 or Arf6 mutants predicted to be in the GDP-bound form (T27N), in the GTP-bound form (Q67L), or missing a myristoylation site (G2A). Binding to PA or PI (4,5)P2 beads was assayed by Nonidet P-40 lysates (Fig. 5A). Whereas binding to PI (4,5)P2 was very low, the four Arf6 proteins differed significantly in their binding to PA. Wild type Arf6 and the T27N GDP-bound mutant showed significant binding to PA that was comparable with endogenous β-cop binding. Binding of the Q67L GTP-bound mutant to PA was more than 15-fold higher, whereas the mutant lacking myristate showed undetectable binding. These results suggest that binding of Arf6 to PA requires myristoylation and depends on the nucleotide state of the protein. In a similar experiment we compared the affinity of the wild type Arf1 and the corresponding mutants with PA (Fig. 5B). Again, binding to PA was totally eliminated for the mutant lacking the myristoylation site (Arf1 G2A). Interestingly, the differences in PA binding between the GTP-bound (Arf1 Q71L) and the GDP-bound (Arf1 T31N) forms of Arf1 were much smaller than the corresponding differences for Arf6. Although interpretation of this result depends entirely on how well the Arf1 proteins retain their nucleotide in comparison with the Arf6 proteins after lysis, it is nevertheless important to note that there is a significant difference between Arf1 and Arf6 with respect to PA binding.

The relative amounts of endogenous β-cop binding to PA beads were unchanged for the four Arf1 proteins (Fig. 5B), indicating that Arf1 and coatomer interact with PA independently of one another and not as a complex. To explore this further, we transfected COS cells with two different amounts of plasmid expressing the active form of Arf1 (Q71L) and assayed recombinant Arf1 and endogenous β-cop binding to PA for all conditions (Fig. 5C). We saw that β-cop binding to PA remained constant, although the amount of Arf Q71L increased as a function of the input plasmid. Because at the highest plasmid concentration more than 50% of the COS cells express the Arf1 Q71L protein as judged by immunofluorescence (data not shown), we expected to detect increased β-cop binding as a function of increased Arf1 Q71L binding if coatomer and activated Arf1 bound to the PA beads as a complex. Because we did not detect any increase in β-cop binding, we conclude from this experiment that Arf1 and coatomer do not bind to PA beads as a complex.

The Inactive Form of NSF Binds to PA—To determine whether NSF binding to PA is direct, recombinant NSF (46) was used in the binding assay (Fig. 6A). In this experiment we also asked whether the nucleotide state of NSF is important for PA binding and whether binding occurs to other lipid beads. No binding of recombinant NSF to PI (4,5)P2 beads or to uncoupled Affi-Gel beads was detected (Fig. 6A, lanes 7 and 8). Binding to PA beads was very dependent on the nucleotide state of the protein. Under conditions where NSF was in the ATP-bound state and unable to hydrolyze the nucleotide, i.e. in the presence of Mg ions and a nonhydrolyzable analog of ATP (ATPγS), binding to PA was undetectable (Fig. 6A, lane 3). Strongest binding to PA was obtained without no nucleotide and in the presence of EDTA (Fig. 6A, lane 6), conditions that presumably restrict NSF to the ADP-bound state. The range of binding affinities when combinations of ATP, ATPγS, EDTA, and Mg ions were used is also consistent with the idea that strongest binding to PA occurs for the ADP conformation of NSF (Fig. 6A, lanes 2, 4, and 5, and data not shown). A hallmark of NSF function is its sensitivity to alkylation by NEM, which is thought to inhibit the ATPase activity of the protein. We examined NSF binding to PA after NEM treatment in the presence of ATPγS, EDTA, or ATPγS plus Mg ions (Fig. 6B). In general, NEM treatment enhanced NSF binding to PA (Fig. 6B, compare lanes 2–4 with corresponding lanes 5–7). More importantly, NEM-treated NSF was fully capable of PA binding even in the presence of ATPγS and Mg (Fig. 6B, compare lane 5 with lane 8). Thus, PA binding of NSF appears to occur when the protein is in an inactive form.
DISCUSSION

We have synthesized and started to use a PA-specific affinity matrix to identify potential protein targets that bind to this lipid. PA-interacting proteins will be downstream of PLD activation and perhaps following acylation of lysophosphatidic acid (20, 21). PI (4,5)P₂ has also been implicated, directly or indirectly, in several pathways of intracellular transport (25), frequently in settings where PA is also a candidate. To differentiate binding partners for these lipids, we have also synthesized and used a PI (4,5)P₂-specific affinity matrix. In this work we concentrate on traffic-related proteins that bound strongly to PA and less well to PI (4,5)P₂.

In designing this reagent, we have assumed that binding of candidate proteins to PA should be of sufficient strength to occur in detergent solution and withstand several washes. We expect that if such proteins show strong binding in detergent solution, their binding to PA in the cellular setting may be comparable if not stronger. In addition, by presenting PA to the candidate proteins in the form of a two-dimensional patch on the agarose matrix, we have hoped to emulate the physiological state (and perhaps the local concentration) of this lipid on cellular membranes.

The PA beads are a novel reagent, and it was important to establish the specificity of protein binding. Firstly, we have shown throughout this work that candidate proteins binding to PA do not bind to PI (4,5)P₂ under conditions where a known PI (4,5)P₂ target (PLCδ) binds to the PI (4,5)P₂ beads. Because the PA and PI (4,5)P₂ beads share the same palmitoyl moiety, binding of candidate proteins to one but not the other may be independent of the structure of the acyl chain, i.e., it is probably to the head group or possibly to the head group because it is restrained by the acyl chain. A second way to address specificity was to use soluble PA as a competitor of candidate protein binding to the PA beads. Using shorter chain analogs of PA—such as dilauroyl or dioctanoyl, we were able to show for all proteins discussed in this work that soluble PA in the range of 50–100 μM was able to reduce binding to the beads by 75–95%.

Competition of the binding reaction using phosphate, glycerolphosphate, or ATP was negative, reinforcing the specificity of candidate proteins to PA. An additional finding that pertains to the specificity of the PA beads is that related proteins (such as coatomer versus the AP-1 and AP-2 coats) show very different binding affinities. Thus, binding to PA, whereas the other coats did not. Arguing for specificity was also the observation that the binding of Arf1 and Arf6 to the PA beads was significantly different, especially with respect to the activation state of the protein. Thus, binding to immobilized PA is not a general phenomenon for all proteins that cycle between membrane and cytosol but rather is restricted to a subpopulation of this group. We are currently addressing the possibility that all...
may be of some influence because the activated form of Arf6 has strong affinity for this lipid. In this context it is interesting that treatment of cells with phorbol esters (which can activate protein kinase C that in turn activates PLD to produce PA) results in Arf6 translocation to the plasma membrane (49).

The significance of the NSF-PA interaction is more difficult to evaluate primarily because this protein is acquiring with time novel and unexpected functions that do not fit readily within a single experimental paradigm. Originally thought to mediate vesicle fusion directly, it was later shown that NSF, through its ATPase activity, functions primarily as a chaperone to maintain unpaired v-SNARE (SNAP receptors) and t-SNARE complexes (50). In addition, a function of NSF in reassembly of Golgi stacks following mitosis was shown not to depend on its ATPase activity (51). Recently NSF was identified as a binding partner of two plasma membrane proteins: β-arrestin, a protein that mediates internalization of G protein-coupled receptors, and the AMPA receptor, an ionotropic glutamate receptor involved in synaptic transmission (52, 53). Our data indicate that the ADP-bound form of purified NSF has good affinity for PA. In the context of its life cycle, this affinity can be relevant either for recruiting NSF to a PA-rich membrane subdomain for a round of activation or for maintaining GDP-bound NSF on PA-containing membranes following ATP hydrolysis for subsequent reactivation. In both of these cases, PA may provide in trans a phosphate group to stabilize ADP-bound NSF.

A single round of protein transport entails cargo concentration and budding from the donor membrane, vesicle movement, and vesicle tethering and fusion with the acceptor membrane. It is interesting that proteins involved in all three of these steps show affinity for PA. This affinity could be relevant for a specific transport step following signal-dependent activation of PLD such as the need to down-regulate by internalization and subsequent degradation an activated receptor. Affinity for PA could also be relevant for all transport steps involving the proteins in question, but this would require that mechanisms of PA formation and consumption underlie those steps. Thus, because PA can be made by basal or signal-stimulated cellular pathways, it is possible that it has evolved into a versatile regulator of membrane transport in housekeeping settings, signal-dependent settings, or both.

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REFERENCES


Fig. 6. Characteristics of NSF binding to PA beads. A, recombinant Myc-tagged NSF (lane 1; 5% of input in the binding reaction) was diluted in base buffer (50 mM Heps, pH 7.6, 90 mM KCl, 0.4% Nonidet P-40, 0.1% bovine serum albumin) and divided into seven equal aliquots. The indicated components were added (100 μM ATP-γ-S, 2 mM MgCl₂ or 2 mM EDTA as required), and the samples were incubated at room temperature for 10 min. At the end of this incubation the samples were returned on ice, and then received 4 mM dithiothreitol to neutralize the NEM. The samples were diluted in base buffer as above and centrifuged to remove any aggregates. The resultant supernatants (shown in lanes 1 and 2 and representing 5% of input in the binding reaction) were divided into three equal aliquots, and the indicated components were added (100 μM ATP-γ-S, 2 mM MgCl₂, or 2 mM EDTA as required) for 10 min at room temperature. At the end of this incubation the samples were returned on ice, and PA beads were added. Following binding for 1 h, washes, SDS-PAGE, and electrotransfer, the blots were probed with anti-Myc antibodies. B, recombinant Myc-tagged NSF was treated for 30 min on ice with 3 mM NEM plus 5 mM dithiothreitol (lanes 2 and 6–8) or with 3 mM NEM plus 5 mM dithiothreitol (lanes 1 and 2–5). Both samples then received 4 mM dithiothreitol to neutralize the NEM. The samples were diluted in base buffer as above and centrifuged to remove any aggregates. The resultant supernatants (shown in lanes 1 and 2 and representing 5% of input in the binding reaction) were divided into three equal aliquots, and the indicated components were added (100 μM ATP-γ-S, 2 mM MgCl₂, or 2 mM EDTA as required) for 10 min at room temperature. At the end of this incubation the samples were returned on ice, and PA beads were added. Following binding for 1 h, washes, SDS-PAGE, and electrotransfer, the blots were probed with anti-Myc antibodies.

these proteins share a common structural determinant for PA binding.

Binding of coatomer to PA beads involved the entire complex and did not require any additional components such as Arf. This observation is consistent with our earlier report that coatomer bound better to artificial liposomes containing PA than to those made in its absence (14). Interestingly, Arf itself (both from cytosolic sources and from tissue culture lysates) also bound to PA beads. Recent work is suggesting a complex set of relationships between coatomer, Arf, and sorting signals on the surface of membranes, leading to the formation of coated vesicles containing specific cargo (47). An important requirement of these models is the need to concentrate on membrane subdomains all of the interacting protein complexes. We suggest that PA, given its good affinity for both coatomer and Arf, is ideally suited to serve as a nucleation site for the initiation of a budding reaction.

The differential binding of Arf6 to the PA beads depending on activation state may be of relevance in explaining the trafficking of this protein. The GDP-bound form of Arf6 is in endosomal compartments, but activation and binding to GTP result in translocation to the plasma membrane (48). How can a protein that is always membrane-bound accumulate on two separate membranes depending on activation state? Our data indicate that the relative PA content of the two membranes
PA-binding Proteins

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