The EMBO Journal Vol.17 No.20 pp.5923–5932, 1998

Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid

Kris Meerschaert, Veerle De Corte, Yvette De Ville, Joël Vandekerckhove and Jan Gettemans[1]

Flanders Interuniversity Institute for Biotechnology (V.I.B.) and Department of Biochemistry, Faculty of Medicine, Universiteit Gent, Ledeganckstraat 35, B-9000 Gent, Belgium

[1]Corresponding author
e-mail: jan.gettemans@rug.ac.be

An extensive survey was carried out for compounds capable of regulating actin-binding proteins in a manner similar to phosphatidylinositol 4,5 bisphosphate (PI 4,5-P_2). For this purpose we developed a sensitive assay involving release of radioactively phosphorylated actin from the fragminP–actin complex. We found that the structurally simplest lysophospholipid, lysophosphatidic acid (LPA), dissociated the complex between fragminP and actin, whereas other lysophospholipids or sphingosine-1-phosphate were inactive. Furthermore, LPA inhibited the F-actin severing activity of human gelsolin, purified from plasma or as recombinant protein, mouse adseverin and *Physarum* fragminP. Dissociation of actin-containing complexes by LPA analyzed by gelfiltration indicated that LPA is active as a monomer, in contrast to PI 4,5-P_2. We further show that binding of LPA to these actin-regulatory proteins promotes their phosphorylation by pp60$^{c\text{-}src}$. A PI 4,5-P_2-binding peptide counteracted the effects mediated by LPA, suggesting that LPA binds to the same target region in these actin-binding proteins. When both LPA and PI 4,5-P_2 were used in combination we found that LPA reduced the threshold concentration at which PI 4,5-P_2 was active. Significantly, LPA promoted the release of gelsolin from barbed actin filaments in octylglucoside-permeabilized human platelets. These results suggest that lysophosphatidic acid could act as an intracellular modulator of actin-binding proteins. Our findings can also explain agonist-induced changes in the actin cytoskeleton that are not mediated by polyphosphoinositides.

Keywords: fragminP/gelsolin/lysophosphatidic acid/pp60$^{c\text{-}src}$

Introduction

Phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2) is considered to be one of the key regulators of the subcortical actin network in eukaryotic cells (Stossel, 1993; Janmey, 1994; Isenberg and Goldman, 1995). PI 4,5-P_2 micelles, or vesicles containing this phospholipid, bind to a variety of actin-binding proteins (ABPs), including all members of the gelsolin family. Most of these contain two conserved sequence segments of ~10 residues which were designated as PI 4,5-P_2 binding motifs (Janmey *et al*., 1992; Yu *et al*., 1992). The interaction between gelsolin and PI 4,5-P_2 *in vivo* has been corroborated by Hartwig *et al*. (1995), demonstrating dissociation of gelsolin from capped actin filaments following thrombin stimulation of platelets. Chellaiah and Hruska (1996) also measured an increased association of phosphoinositides with gelsolin in osteoclasts that were stimulated with osteopontin. In both cases, the amount of F-actin following stimulation rose significantly, in agreement with the idea that PI 4,5-P_2 uncaps gelsolin from the barbed ends, thus creating conditions favorable for growth at the (+) ends. This phenomenon is tightly connected with activation of the small GTP-binding protein Rac, most likely through stimulation of phosphoinositide kinase activities (Hartwig *et al*., 1995).

We recently showed that PI 4,5-P_2 stimulates phosphorylation by pp60$^{c\text{-}src}$ of a number of ABPs such as gelsolin and CapG (De Corte *et al*., 1997). Tyrosine phosphorylation of ABPs could possibly control their actin-binding properties, but also other functions such as phosphoinositide binding, accessibility for hydrolysis by phospholipase C (Goldschmidt-Clermont *et al*., 1990) or affinities for other target proteins such as phosphatidylinositol 3-kinase (Singh *et al*., 1996), thereby providing a link between pp60$^{c\text{-}src}$ activity and actin organization. Next to PI 4,5-P_2, other phospholipids such as PI 3,4-P_2 and PI 3,4,5-P_3 bind to gelsolin and profilin and display an even higher affinity for profilin than PI 4,5-P_2 (Lu *et al*., 1996). This points to a connection with PI 3-kinase activity and may suggest a hierarchical order in binding of ABPs to different phosphoinositides.

Several studies (Bengtsson *et al*., 1988; Eberle *et al*., 1990) have shown that agonist-induced actin rearrangements can occur independently of changes in the level of phosphoinositides. It has even been reported that EGF stimulation of A431 cells resulted in a decrease of phosphoinositides concomitant with actin polymerization (Dadabay *et al*., 1991). In these cases phospholipids are unlikely to be involved in uncapping of actin-binding proteins from barbed ends. Lin *et al*. (1997) suggested that the higher affinity of Ca^{2+}-gelsolin for phosphoinositides could compensate for the observed decrease in phosphoinositides. We followed a different approach and carried out an exhaustive screening procedure for molecules which are able to dissociate ABP–actin complexes, in order to find out whether other compounds might be implicated in regulating ABP–actin interactions. For this we used the ^{32}P-labeled fragminP–actin complex from *Physarum polycephalum* (Gettemans *et al*., 1992, 1993). Dissociation of the complex by various compounds was surveyed by measuring ^{32}P-labeled actin in the supernatant after immunoprecipitation with affinity-purified anti-fragminP antibodies.

© Oxford University Press

We discovered that lysophosphatidic acid (LPA) specifically dissociated the fragminP–actin complex, and other actin–ABP complexes, as efficiently as did PI 4,5-P_2. In addition, LPA stimulated pp60$^{c\text{-}src}$-mediated phosphorylation of several actin-binding proteins including gelsolin and adseverin. Our findings suggest a dual mediator role for LPA, as was recently shown for sphingosine-1-phosphate, and we discuss how LPA activity could participate in current models describing actin organization.

Results

Lysophosphatidic acid dissociates the 1:1 EGTA-resistant complexes of actin with proteins of the gelsolin family

We used fragminP–actin to screen for dissociation because it can be radiolabeled specifically at Thr203 of the actin subunit (Gettemans *et al.*, 1992). Dissociation of phosphorylated actin from the complex allowed fast and accurate measurement of the amount of ^{32}P-actin in the supernatant after fragminP immunoprecipitation. Figure 1A lists some of the compounds used in this experiment. Only PI 4,5-P_2, PI 4-P and LPA were highly active, releasing up to 80–90% of radioactively labeled actin from the fragminP–actin complex. Lysophosphatidylserine (LPS) displayed a much weaker activity. Other (phospho)lipid or lysophospholipid analogs such as lysophosphatidylethanolamine (LPE), phosphatidic acid (PA), ceramide (Cer) and arachidonic acid (AA) showed no effect (Figure 1A). In addition, lysophosphatidylcholine and lyso-platelet activating factor (lyso-PAF) were equally inactive (data not shown). It has been speculated that sphingosine-1-phosphate (S-1-P) could physically interact with actin-binding proteins (Yamamura *et al.*, 1996). In our assay, S-1-P was poorly active and yielded only a maximal dissociation of 10%.

The interaction between fragminP and LPA was studied in further detail by measuring the degree of dissociation of a fixed concentration of the complex (0.4 μM) versus gradually increasing LPA concentrations. Only limited dissociation was measured at low (20 μM) and intermediate (40 μM) LPA concentrations (Figure 1B). Further addition of LPA caused an abrupt increase in activity leading to full separation of the proteins at 250-fold molar excess of LPA (100 μM). When PI 4,5-P_2 was used, we noticed a more gradually increasing dissociation, proportional to the amount of PI 4,5-P_2 (Figure 1B).

We also examined whether the phosphorylated fragminP–actin complex was equally sensitive to LPA as its unphosphorylated form. Therefore, we measured dissociation of the latter by SDS–PAGE analysis of the anti-fragminP immunoprecipitate. As for the phosphorylated complex we observed only limited dissociation at low LPA concentrations, followed by a steep increase at higher LPA concentrations (data not shown).

The dissociation of fragminP–actin was also analyzed by gel filtration. For this purpose it was more convenient to use the naturally occurring complex between actin and the N-terminal half of fragminP, termed fragmin$_{19}$ (De Corte *et al.*, 1996), because dissociation of the complex yields products that are easily separated by gel filtration. In addition, it allowed us to investigate whether this fragminP domain contains a binding site for LPA (it lacks the second PI 4,5-P_2-binding site but contains the first binding site). When the A-F_{19} complex (5.5 μM) was chromatographed through a Superdex gel filtration column after preincubation with a 200-fold molar excess of LPA, the protein peak corresponding with A–F_{19} dissociated into three peaks (Figure 1C, right panel). Analysis of the column eluate by SDS–PAGE revealed that the fractions corresponding with the first peak contained F-actin (Figure 1C, insets), due to the salt present in the elution buffer. Since actin was able to polymerize we conclude that it was not denatured (this was also confirmed by native gel electrophoresis; data not shown). The second peak corresponded with undissociated A-F_{19}, eluting with an apparent M_r of 60 000, whereas the last peak contained only fragmin$_{19}$. Chromatography in the presence of PI 4,5-P_2 caused the A–F_{19} complex to shift to a higher M_r (not shown).

In contrast to PI 4,5-P_2, LPA does not form micelles under the conditions used due to its high CMC (1.3 mM; Jalink *et al.*, 1990). This explains why fragmin$_{19}$ eluted from the column with an apparent M_r corresponding to its determined molecular mass of 19 123 Daltons (De Corte *et al.*, 1996). Furthermore, experiments using the dye 6-(*p*-toluidino)2-naphtalenesulfonate (TNS) (Das and Hajra, 1992) demonstrated that LPA, in our buffer conditions and used at the indicated concentrations, was present as a monomer (data not shown). Lack of full dissociation of the actin–fragmin$_{19}$ complex can be ascribed to re-association of both components during chromatography, in combination with dilution of the lipid during buffer elution. Alternatively, it may reflect weaker binding of LPA to fragmin$_{19}$ in comparison with intact fragminP. The latter hypothesis seems more likely since LPA and PI 4,5-P_2 bind to the same target regions in actin-binding proteins (see below).

The studies on gelsolin–LPA interaction were carried out with plasma gelsolin in complex with skeletal muscle actin, and the immunoprecipitate obtained with monoclonal anti-gelsolin antibodies was analyzed by SDS–PAGE. Addition of LPA at low concentrations (20 μM) resulted in limited dissociation of actin; when the LPA concentration was further increased (>40 μM) nearly 100% dissociation was measured (Figure 2A). This finding agrees with our observations on the fragminP–actin complex (Figure 1B), suggesting that LPA acts in a similar manner on both proteins.

The effect of LPA on the F-actin severing activity of plasma gelsolin and other ABPs was measured using a fluorimetric assay. Severing of F-actin filaments by plasma gelsolin results in the formation of many pointed ends, causing fast depolymerization of F-actin and consequently a drastic decrease in the relative fluorescence of labeled F-actin. Preincubation of 25 nM plasma gelsolin with 6.25 μM LPA caused complete inhibition of F-actin severing activity, as for PI 4,5-P_2 (Figure 2B). Recombinant cytosolic gelsolin gave the same results (data not shown). The effect of LPA on gelsolin could be partially reverted by treating the sample with phospholipase B; as for PI 4,5-P_2, addition of Triton X-100 (Janmey and Stossel, 1987) counteracted the effect of LPA (Figure 2B). These data show that the ABP is not irreversibly denatured by LPA. A very similar dose-dependent inhibition of F-actin severing activity by LPA was also recorded for

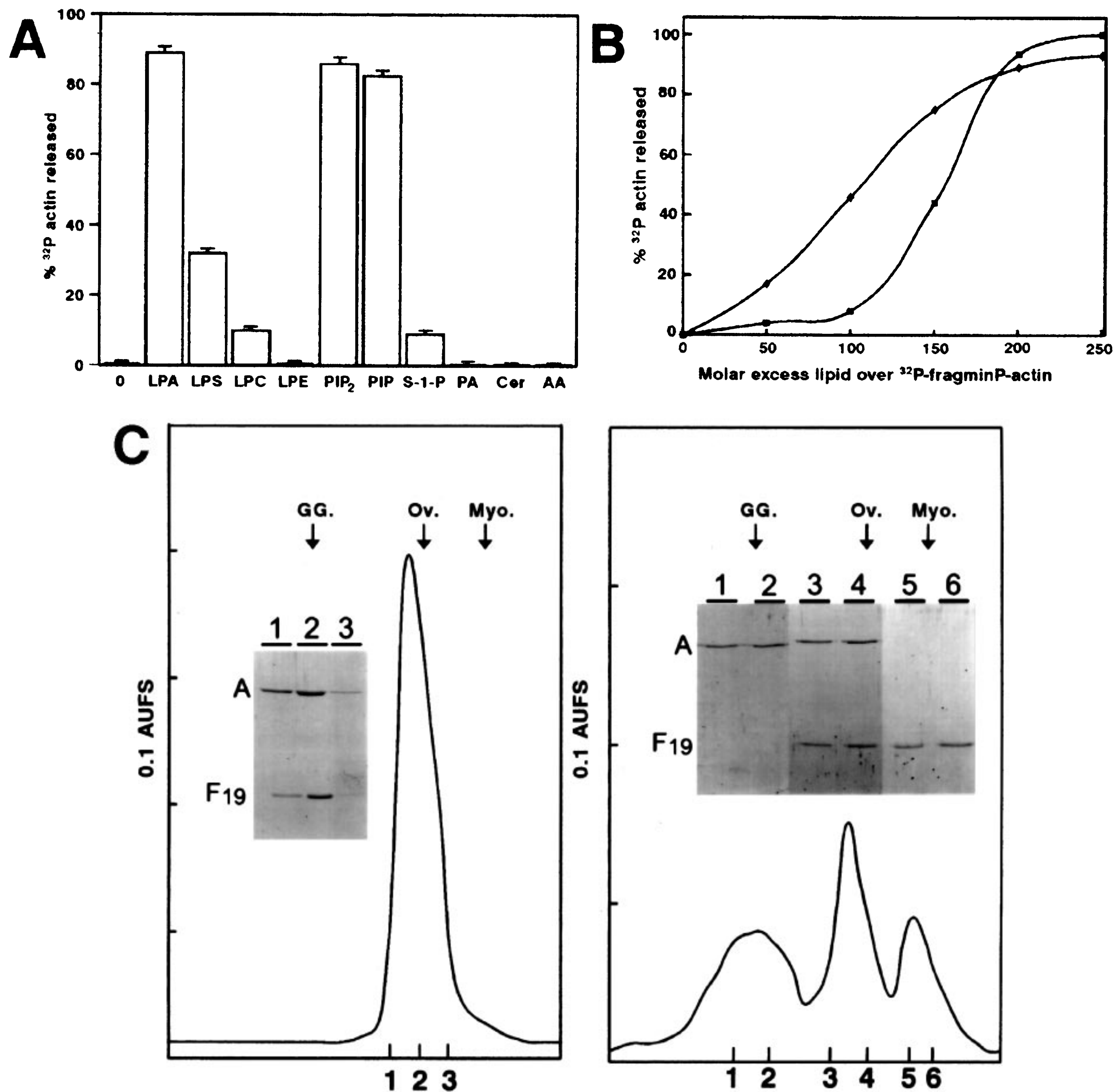

Fig. 1. Dissociation of ^{32}P-labeled fragminP–actin complex by LPA and PI 4,5-P_2. (**A**) Quantification of fragminP–actin dissociation by LPA and comparison with other phospholipids. ^{32}P-labeled fragminP–actin was incubated with the lipids (250-fold molar excess over phosphorylated complex = 100 μM) and subsequently immunoprecipitated with anti-fragminP antibodies and protein G–Sepharose. The supernatant was counted in a scintillation counter. The *y*-axis shows the percentage of actin released from the complex. AA, arachidonic acid; PA, phosphatidic acid; Cer, C_6-ceramide; S-1-P, sphingosine-1-phosphate. (**B**) Dose–response curve of LPA and PI 4,5-P_2. The experiment was performed in the same way as described in (A). LPA activity (■) increases dramatically above a threshold concentration of 100-fold molar excess (40 μM). PI 4,5-P_2 activity (◆) increases progressively with higher concentrations. (**C**) LPA is present as a monomer upon gel filtration with actin-fragmin$_{19}$. Left panel, control: actin-fragmin$_{19}$ elutes as a 60 kDa complex. Right panel: as (A) but after the addition of LPA. Note the appearance of a new peak eluting with an apparent M_r of 20 kDa. Insets: SDS–PAGE analysis of the fractions: the third peak corresponds with fragmin$_{19}$. A, actin; F_{19}, fragmin$_{19}$. Arrows indicate the elution position of calibration markers: GG., gamma globulin (158 kDa); Ov., ovalbumin (44 kDa); Myo., myoglobin (17 kDa).

recombinant murine adseverin (Figure 2C) and recombinant fragminP (data not shown). These experiments illustrate the specific effect of LPA on different members of the gelsolin family.

Experiments involving quenching of intrinsic tryptophane fluoresence through lipid binding by fragminP (in the absence of calcium) were employed to derive an apparent dissociation constant for LPA, as was done previously for the CapG/PI 4,5-P_2 interaction (Lin *et al.*, 1997). Optimal fitting of the data was accomplished when two binding sites were considered. We calculated a K_d value of 28.9 μM for LPA (correlation coefficient 0.9967), whereas a value of 14.1 μM was obtained for PI 4,5-P_2 (correlation coefficient 0.9994).

To exclude the possibility that the LPA sample was contaminated we analyzed the lipid by electrospray–ionization mass spectrometry. Only one peak was present in the mass-transformed spectrum (data not shown), corresponding with a molecular mass of 435 Da, in agreement with the M_r of lysophosphatidic acid with a single positive charge. We therefore conclude that the observed effect is due solely to LPA.

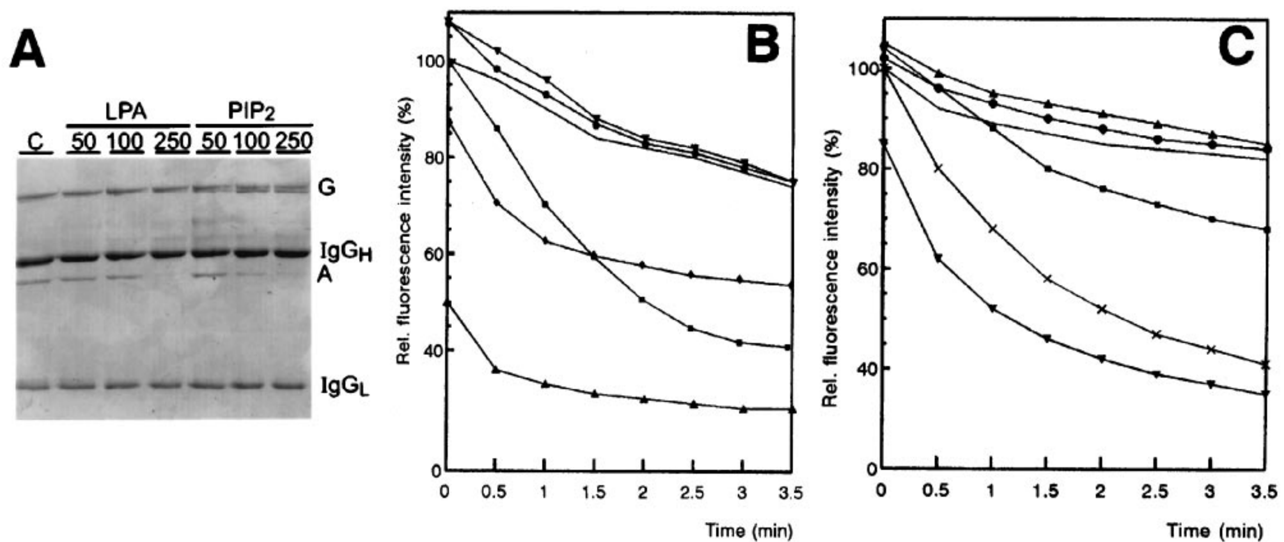

Fig. 2. Dissociation of 1:1 gelsolin–actin by lysophosphatidic acid and modulation of gelsolin and adseverin F-actin severing activity by LPA. (**A**) Dissociation of gelsolin–actin by LPA and comparison with PI 4,5-P_2. Gelsolin–actin complex (0.4 μM) was preincubated with the indicated molar excess (top) of LPA or PI 4,5-P_2 over actin–gelsolin complex. Pellets were analyzed on SDS–PAGE gels followed by Coomassie Blue staining. C, control: gelsolin–actin immunoprecipitated in the absence of lipids. G, gelsolin; A, actin. IgG_H and IgG_L refer to the IgG heavy and light chains, respectively. (**B**) LPA-mediated inhibition of F-actin severing by gelsolin is partially restored by Triton X-100 or phospholipase B. Severing of pyrene-labeled actin filaments with 25 nM gelsolin (▲) is inhibited in the presence of 6.25 μM LPA (●). Treatment of the gelsolin–LPA mixture with 1 U phospholipase B for 30 min at 37°C (◆). Addition of 0.25% Triton X-100 partially restores severing activity of gelsolin (■). Inhibition of gelsolin by 6.25 μM PI 4,5-P_2 (▼). Control: depolymerization of 400 nM F-actin without gelsolin (—). (**C**) Inhibition of the severing activity of mouse adseverin by LPA: 50 (1.25 μM; ×), 100- (2.5 μM; ■) and 250-fold (6.25 μM; ▲) molar excess and 250× PI 4,5-P_2 (●). (▼) adseverin activity in the absence of lipid; (—) control, no adseverin.

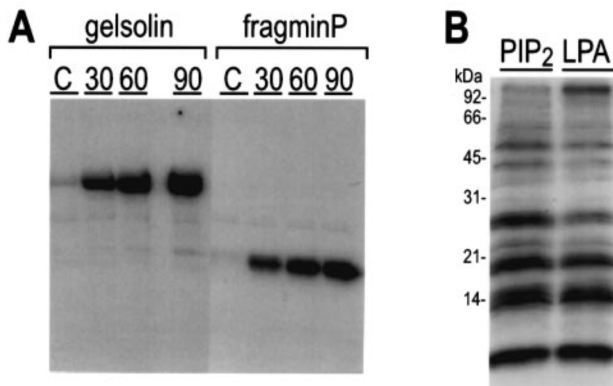

Fig. 3. LPA-mediated stimulation of gelsolin and fragminP phosphorylation by $pp60^{c\text{-}src}$. (**A**) Autoradiogram showing phosphorylation of gelsolin and fragminP by $pp60^{c\text{-}src}$ in the presence of a 30- (75 μM), 60- or 90-fold molar excess of LPA. Exposure time was 2 h at room temperature. C, control; phosphorylation in the absence of LPA. (**B**) Phosphopeptide pattern after gelsolin phosphorylation by $pp60^{c\text{-}src}$ in the presence of a 60-fold molar excess of PI 4,5-P_2 (left) or LPA (right). After phosphorylation and digestion with endolysine-C, the peptides were separated on a 20% polyacrylamide gel. M_r markers are indicated on the left. Exposure time was overnight at room temperature.

Lysophosphatidic acid stimulates $pp60^{c\text{-}src}$-mediated phosphorylation of PI 4,5-P_2-binding ABPs

In a previous report we demonstrated that PI 4,5-P_2 stimulates phosphorylation of gelsolin and related ABPs by $pp60^{c\text{-}src}$ (De Corte *et al*., 1997). In view of the results with LPA, we studied the effect of this lysophospholipid on the phosphorylation of gelsolin and fragminP (Figure 3A). Estimations of the extent of ^{32}P-incorporation revealed an 18- to 20-fold increase of label for fragminP and gelsolin when LPA was present in 60- to 90-fold molar excess (Figure 3A). From a reciprocal Lineweaver–Burk plot we calculated a K_m value of ~5 μM for phosphorylation of gelsolin by $pp60^{c\text{-}src}$ in the presence of a 60-fold molar excess LPA. Apart from PI 4,5-P_2 and PI 4-P (not shown), LPA was the only compound that promoted a significant increase in phosphorylation. No stimulatory effect on the phosphorylation of RCAM-lysozyme was observed (not shown), indicating that LPA did not modulate $pp60^{c\text{-}src}$ activity but induces phosphorylation through binding with the ABP. We also investigated whether the sites phosphorylated in the presence of PI 4,5-P_2 or LPA were similar. For this we compared the autoradiograms of both ^{32}P-labeled peptide patterns obtained after SDS–PAGE analysis of partial endolysine-C digests. Figure 3B reveals a highly similar, if not identical, phospho-peptide fingerprint in accordance with modification of an identical residue(s).

The effects of lysophosphatidic acid are reverted by a peptide that specifically binds to phosphatidylinositol 4,5 bisphosphate

Previous work has led to the delineation of two short segments in gelsolin and other actin-binding proteins that interact with PI 4,5-P_2 (Janmey *et al*., 1992; Yu *et al*., 1992; T'Jampens *et al*., 1997). The corresponding synthetic peptides inhibit the effect of PI 4,5-P_2 on the actin-binding properties of gelsolin. When the villin peptide 133-YNVQRLLHVKGKKNVV-148 was added to LPA prior to incubation with gelsolin, no inhibition of the F-actin severing activity was observed (Figure 4A). Similarly, when the peptide was added in a 2-fold molar excess over LPA after incubating gelsolin with LPA, we noticed a partial recovery of the severing activity

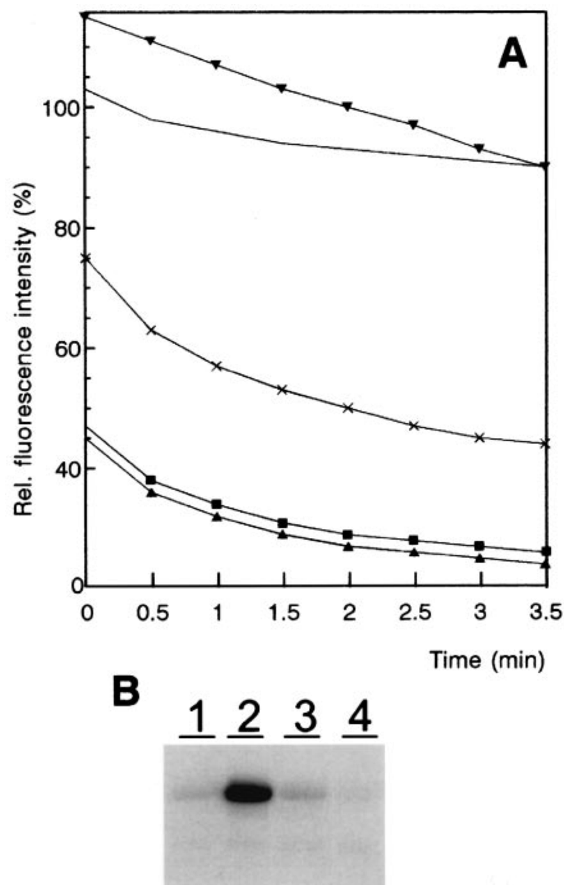

Fig. 4. The PI 4,5-P_2 binding peptide derived from villin reverses the effect of LPA on gelsolin. (**A**) Gelsolin (25 nM) F-severing activity (▲) and inhibition in the presence of 6.25 μM LPA (▼). Treatment of the gelsolin–LPA mixture with a 2-fold molar excess, relative to LPA, of villin peptide partially restores severing activity of gelsolin (×). Preincubation of LPA with the villin peptide (2-fold excess over LPA) completely abolishes inhibition of the severing activity by LPA (■). Control: depolymerization of 400 nM F-actin without gelsolin (—). (**B**) LPA-mediated stimulation of gelsolin phosphorylation by $pp60^{c\text{-}src}$ is counteracted by the villin peptide. Phosphorylation with $pp60^{c\text{-}src}$ in the absence (lane 1) or presence (lane 2) of 150 μM (60-fold molar excess) of LPA. Lane 3: addition of a 2-fold molar excess of villin peptide, relative to LPA, to the gelsolin–LPA mixture and subsequent phosphorylation. Lane 4: preincubation of the same amount of villin peptide with LPA prior to mixing with gelsolin and $pp60^{c\text{-}src}$.

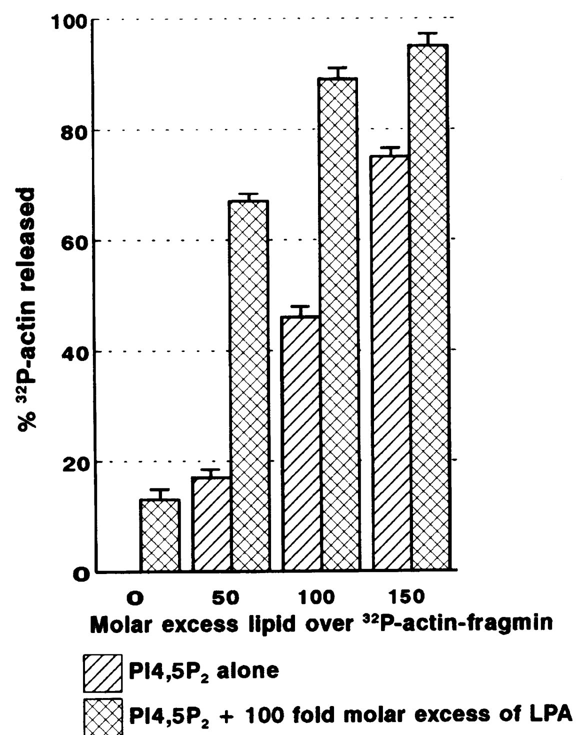

Fig. 5. Co-operative effect of LPA and PI 4,5-P_2 on the dissociation of ^{32}P-labeled fragminP–actin. The experiment was performed in the same manner as described in Figure 1. In all samples a constant amount of LPA was present (40 μM). Subsequently PI 4,5-P_2 was added (molar excess indicated in the *x*-axis) and fragminP was immunoprecipitated. Note that, particularly at lower PI 4,5-P_2 concentrations, the effect of both compounds together (double hatched bars) is more pronounced than when PI 4,5-P_2 is added alone (single hatched bars).

(Figure 4A). Similar results were obtained using fragminP or mouse adseverin (data not shown).

When the villin peptide was added to the LPA–gelsolin mixture at an equal molar ratio relative to LPA, phosphorylation of gelsolin by $pp60^{c\text{-}src}$ decreased dramatically (Figure 4B, lane 3). Similarly, when the villin peptide was incubated with LPA prior to the addition of gelsolin, no phosphorylation was observed (Figure 4B, lane 4). These results suggests that the LPA and PI 4,5-P_2 binding sites on ABPs are very similar and perhaps identical.

LPA and PI 4,5-P_2 co-operatively inhibit actin-binding proteins

All data shown above support the conclusion that both LPA and PI 4,5-P_2 act similarly on a number of PI 4,5-P_2-sensitive actin-binding proteins. This led us to hypothesize that both components could display co-operative effects. As a result, LPA could modulate the activity of PI 4,5-P_2 by lowering the threshold concentration for inhibition of ABPs. To test this possibility, we measured the dissociation of the ^{32}P-labeled fragminP–actin complex using increasing amounts of PI 4,5-P_2 under conditions where a constant amount of LPA was present. In the absence of PI 4,5-P_2 we measured only ±15% dissociation (Figure 5), in agreement with data shown earlier (Figure 1B). However, upon addition of relatively small amounts of PI 4,5-P_2 we measured a drastic increase of dissociation which was much higher than in the presence of PI 4,5-P_2 alone (i.e. from 17 to 65% or from 45 to 90% upon addition of 20 or 40 μM PI 4,5-P_2, respectively). These data afford a strong argument that LPA and PI 4,5-P_2 could act in a co-operative manner in controlling the activity of PI 4,5-P_2-regulated actin-binding proteins.

Lysophosphatidic acid uncaps gelsolin from the barbed ends of actin filaments in human platelets

We investigated the potential *in vivo* role of lysophosphatidic acid by treating permeabilized human platelets with LPA, as was done previously with PI 4,5-P_2 (Hartwig *et al.*, 1995; Barkalow *et al.*, 1996). Octylglucoside (0.4%) treatment of platelets only causes formation of small holes (20–100 nm diameter) and leaves the plasma membrane largely intact (Hartwig *et al.*, 1995). Resting platelets were used for our experiments because >95% of the filaments are capped by proteins like gelsolin and capping protein (Hartwig *et al.*, 1995). When phospholipids are added to

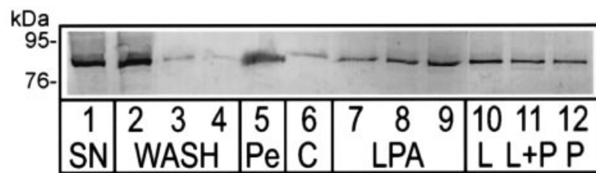

Fig. 6. LPA displaces gelsolin from actin filaments in permeabilized human platelets. Octylglucoside (0.4%) treated platelets were washed repeatedly with buffer to remove free gelsolin. The supernatants were electrophoresed, blotted and probed for gelsolin (lane 2, first wash; lane 3, third wash; lane 4, fifth wash). Lane 1 represents a total extract of untreated platelets (control). Most gelsolin is retained in the permeabilized platelets (lane 5). The samples were then treated with either buffer alone (lane 6) or buffer with 100 μM LPA (lane 7), 200 μM LPA (lane 8) or 400 μM LPA (lane 9). Note that the apparent higher release of gelsolin in lane 6 in comparison with lane 4 is due to resuspension of the sample in a much smaller volume. Lane 10, 200 μM LPA; lane 11, 100 μM LPA + 100 μM PI 4,5-P_2; lane 12, 200 μM PI 4,5-P_2.

permeabilized platelets they uncap gelsolin and capping protein from the (+) ends of cytosolic actin filaments and this can be followed by Western blotting. Although some variability has been reported as to the amount of gelsolin that is released upon phospholipid treatment (1–25%, Barkalow *et al*., 1996), this assay provides a valuable tool to analyze the activity of phospholipids on actin-binding proteins *in vivo*. We used a similar approach to study the effect of LPA on gelsolin uncapping from actin filaments.

Permeabilized platelets were washed repeatedly to remove unbound gelsolin (Figure 6, lanes 2–4). When LPA (100 μM) was added we noticed an increased staining for gelsolin in comparison with the control (Figure 6, lanes 6 and 7–9). Since LPA does not cause membrane leakiness, even at very high concentrations (500 μg/ml; Jalink *et al*., 1990), this finding suggests that LPA uncaps gelsolin from barbed ends of actin filaments. PI 4,5-P_2 also released gelsolin from platelets (Figure 6, lane 12). Comparison between PI 4,5-P_2 and LPA showed that LPA was at least as effective as PI 4,5-P_2 (Figure 6, compare lane 10 with lane 12). Other compounds such as phosphatidylserine and lysophosphatidylinositol showed no effect whereas lysophosphatidylserine displayed a weak effect (not shown), in agreement with earlier findings (Figure 1A). Addition of both LPA and PI 4,5-P_2 resulted in an intermediate uncapping activity (Figure 6, lane 11).

Discussion

We have demonstrated that lysophosphatidic acid inhibits the formation and induces the dissociation of complexes between actin and actin-binding proteins. The proteins used in this study (human plasma gelsolin, recombinant human cytosolic gelsolin, *Physarum* fragminP and mouse adseverin) are also regulated by PI 4,5-P_2. In addition, their phosphorylation by pp60$^{c\text{-}src}$ was dramatically stimulated in the presence of LPA. Based on structural and functional homologies among members of the gelsolin family, we predict that severin (André *et al*., 1988), mammalian CapG (Yu *et al*., 1990) and villin (Arpin *et al*., 1988) are also regulated by LPA. Tight association between lysophospholipids and highly purified preparations of plasma gelsolin was previously reported by Janmey *et al*. (1987), indicating that these compounds are associated with gelsolin in a physiological environment. In addition to the similar effects on their target proteins, the activities of LPA and PI 4,5-P_2 were equally inhibited by a synthetic villin peptide known to bind PI 4,5-P_2 specifically (Arpin *et al*., 1988; Gettemans *et al*., 1995). This adds further support to the idea that both lipids act in a very similar manner on different members of this family of actin-binding proteins.

When a detailed quantitative comparison between PI 4,5-P_2 and LPA was made, we noticed particular differences in the dose–response curve. While PI 4,5-P_2 showed a linear progression in dissociation of ABP–actin complexes, reaching full activity at 100 μM, LPA followed a sigmoidal curve, with a steep rise in activity at concentrations >40 μM. PI 4,5-P_2 is known to act in the form of micelles, whereas LPA remains monomeric due to its high critical micelle concentration (Jalink *et al*., 1990). It is therefore unlikely that the sigmoidal curve is due to the action of micelles that are formed at elevated LPA concentrations. The steep rise in activity is not to be ascribed to a detergent-like effect since actin retained its ability to polymerize, and the inhibitory effect of the actin-binding proteins could be counteracted by phospholipase B, a synthetic PI 4,5-P_2 binding peptide or Triton X-100. A similar curve could also be explained by assuming that distinct LPA binding sites exist on ABPs, acting in a co-operative or sequential manner. This hypothesis requires more detailed analysis in the future.

To our knowledge this is the first report documenting a specific interaction between a lysophospholipid and members of the gelsolin family and this finding raises intriguing questions concerning the structural requirements for phospholipids that bind to and inhibit actin-binding proteins. Apart from PI 3,4 P_2, PI 3,4,5 P_3 (Lu *et al*., 1996), PI 4,5-P_2 and to a lesser extent PI 4-P, no other phospholipids with such properties were described so far. The former may suggest a functional link between the PI 3-kinase pathway and the organization of the subcortical actin network, but considering their low abundance in stimulated cells it is believed they do not play a direct role in the regulation of actin assembly, except in a few specific cases (Toker and Cantley, 1997). Interestingly, phosphatidic acid, arising through incorporation of a second acyl chain at the sn-2 position of the glycerol moiety of LPA, is completely inactive. It thus appears that, for the active molecules, the common minimal structural requirement consists of one phosphate group per acyl chain.

The key question is if LPA can be considered as a candidate intracellular regulator of the actin cytoskeleton. Our finding that LPA uncaps gelsolin from actin filaments in permeabilized human platelets with the same efficiency as PI 4,5-P_2, and the reported intracellular LPA concentrations (as high as 80 μM, see below) strongly support this idea. LPA is known primarily for its hormone-like and mitogenic activities and activates its own G-protein coupled receptor (Moolenaar *et al*., 1997). cDNAs coding for such a receptor have been identified in *Xenopus* (Guo *et al*., 1996), mouse (Hecht *et al*., 1996) and man (An *et al*., 1997). However, the concentration ranges where LPA displays biological activity *in vivo* are very diverse, varying from nanomolar (receptor activation), suggesting high affinity interactions, to micromolar (mitogenesis),

suggesting medium-affinity interactions. Furthermore, as pointed out earlier (Moolenaar, 1995), the commonly held theory of LPA-mediated receptor activation has not been verified in all cases and in this respect the mitogenic effect of LPA requires closer scrutiny. Also, LPA interferes directly with a number of intracellular components that play a role in signal transduction. It interacts with n-chimaerin (Ahmed *et al*., 1993) and regulates the interaction between G-proteins and GAPs (Tsai *et al*., 1989; Chettibi *et al*., 1994). Furthermore, glycosylphosphatidylinositol-specific phospholipase D (Low and Huang, 1993) and phosphatidylinositol 3-kinase (Lauener *et al*., 1995) are inhibited by PA and LPA, whereas protein kinase C is stimulated by LPA (Sando and Chertihin, 1996). These results, combined with our findings, support the concept of a dual signaling activity of LPA, either through activation of an LPA receptor or as a secondary messenger.

In this respect it is worth mentioning a number of reports showing a similar dual signaling role for sphingosine-1-phosphate (S-1-P). S-1-P was originally characterized as a second messenger molecule, promoting DNA synthesis in Swiss 3T3 fibroblasts (Olivera and Spiegel, 1993). Zhang *et al*. (1995) showed that sphingosine-1-phosphate is produced in response to stimulation of 3T3 fibroblasts, eliciting calcium release from IP_3-sensitive and -insensitive pools. Lee *et al*. (1998) cloned a receptor for S-1-P (Edg-1) that mediates the extracellular effects of S-1-P but a major breakthrough was provided by Meyer zu Heringdorf (1998) who showed that calcium release could be mediated only by microinjection of S-1-P. Other data emphasizing intracellular or extracellular modes of action of S-1-P (Rani *et al*., 1997; Wang *et al*., 1997; Yatomi *et al*., 1997; Cuvillier *et al*., 1998) are probably more indicative of the exceptionally broad biological diversity of S-1-P rather than reflecting seemingly contradictory data. Indeed, Van Brocklyn (1998) recently demonstrated that mitogenesis and prevention of apoptosis (two typical S-1-P responses) are unrelated to binding to Edg-1. S-1-P suppresses apoptosis of HL-60 and PC12 cells, but these cells do not show expression of Edg-1 mRNA. These authors demonstrate that S-1-P is a prototype for a novel class of lipid mediators that act both extracellularly as ligands for cell surface receptors and intracellularly as second messengers. We provide evidence that LPA may have an intracellular role. Another lipid that is characterized by a dual role is Platelet Activating Factor (PAF). PAF activates its cognate receptor but there is also evidence for intracellular synthesis of PAF and cytosolic binding sites in neutrophils (Svetlow and Nigam, 1993).

In thrombin-stimulated platelets, the LPA concentration reaches intracellular values as high as 47 μM (Watson *et al*., 1985), or even 77 μM (Benton *et al*., 1982). Half maximal activity was observed at ~60 μM in our *in vitro* assays (Figure 1B). LPA is also released from platelets (measured after 10 min; Eichholtz *et al*., 1993). This is possibly associated with leakage of the cells which occurs during aggregation, because production of LPA in a lipid environment is not necessarily followed by release into the surrounding medium. For example, challenging erythrocytes with calcium causes shedding of microvesicles that contain LPA; the lysophospholipid remains unchanged in the vesicles but could be extracted with BSA (Fourcade *et al*., 1995). Although few data are available for other cells, these findings demonstrate that intracellular LPA concentrations can rise temporarily to values which were found to be active in our experiments.

Although future *in vivo* experiments have to confirm our hypothesis of a secondary messenger role for LPA in actin organization, the information currently available does not exclude such a mechanism and could even explain contradictory findings. Indeed, actin polymerization in thrombin-stimulated platelets and an increase in the intracellular LPA concentration (Watson *et al*., 1985) are known to occur within a similar time span (60–75 s) as the exposure of barbed filament ends by an uncapping mechanism and subsequent assembly of actin (Hartwig *et al*., 1995). Although D3 and particularly D4 polyphosphoinositides are considered as the universal agents in this mechanism, Dadabay *et al*. (1991) showed that stimulation of A431 cells with EGF also resulted in the dissociation of actin–gelsolin complexes and subsequent F-actin polymerization, but that PI 4-P and PI 4,5-P_2 were not involved in this mechanism. Our results allow us to propose a role for LPA in this process because stimulation of fibroblasts with epidermal growth factor causes PLA_2 activation (Peppelenbosch *et al*., 1992 and references therein). Through this activity, LPA (Fukami and Takenawa, 1992) and arachidonic acid are generated. LPA thus generated could be implicated in the temporal and spatial control of membrane ruffling and changes in cell morphology. Moreover, Arcaro (1998) found that Rac indirectly promotes dissociation of 1:1 actin–gelsolin complexes in neutrophils through a signaling pathway that is independent of PI 4,5-P_2 or PI 3,4,5-P_3. These and other data show that uncapping of gelsolin from actin filaments can occur by mechanisms other than changes in the level of polyphosphoinositides.

In conclusion, we have presented evidence that a well known and widely occurring lysophospholipid inhibits actin-binding proteins of the gelsolin family. This finding broadens the field of compounds susceptible of regulating the actin cytoskeleton and may extend our conceptual understanding of the different pathways by which actin polymerization can be controlled.

Materials and methods

Preparation of lipids

L-α-lysophosphatidic acid, 1-oleoyl (C_{18}:1 [*cis*] –9) and other (lyso)phospholipids were obtained from Sigma (St Louis, USA). All lipids were dissolved in water at a concentration of 1 mM (or 5 mM), except for C_6-ceramide which was dissolved in ethanol. S-1-P was dissolved in methanol–water (1:1 v/v) at a concentration of 2 mM. Suspensions were stored at –80°C until further use.

Proteins

Actin was prepared from rabbit skeletal muscle according to Spudich and Watt (1971), further purified by Sepharose 200 gel-filtration and kept in G-buffer (2 mM Tris–HCl pH 7.6, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, and 0.02% NaN_3). Pyrene-labeled actin was prepared according to Brenner and Korn (1983). The native fragminP–actin complex, $fragmin_{19}$-actin and recombinant fragminP were purified as described previously (Gettemans *et al*., 1993; De Corte *et al*., 1996; T'Jampens *et al*., 1997). Human plasma gelsolin was isolated as described by Cooper *et al*. (1987). Recombinant cytosolic gelsolin was cloned as a *Bam*HI fragment in the pET11 expression vector and, after transformation and expression in *Escherichia coli*, purified from the cytosol through DEAE–ion exchange chromatography. Recombinant mouse adseverin was purified as described by Robbens *et al*. (1998). The 1:1 actin–gelsolin complex was reconstituted *in vitro*

by incubating appropriate quantities of actin and gelsolin on ice in the presence of 2 mM $CaCl_2$ for 2 h, followed by the addition of 5 mM EGTA. Further purification was done by chromatography through a Sephacryl 200 gel-filtration column, equilibrated with TEDA buffer (10 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM dithiothreitol, 0.02% NaN_3). Phospholipase B was from Sigma (St Louis, USA). Recombinant human $pp60^{c\text{-}src}$ kinase was obtained from Upstate Biotechnology Incorporated (UBI, New York, NY).

Actin polymerization assays (fluorescence spectroscopy)

All fluorescence measurements were performed as described (Gettemans *et al*., 1995). FragminP, gelsolin or adseverin (25 nM) were used for routine severing assays. Lysophospholipids were preincubated with the ABP for 5–10 min. Subsequently the mixture was added to the pyrene-labeled F-actin solution (8 μM stock solution, 25% pyrene-labeled) and the decrease in fluorescence was measured over time.

Immunoprecipitation and Western blotting

Purified native fragminP–actin (0.4 μM) or muscle actin–gelsolin (0.4 μM) complexes were preincubated with various amounts of (lyso)-phospholipids for 30 min and then further incubated for 2 h with anti-fragminP antibodies (Gettemans *et al*., 1995; De Corte *et al*., 1996) or anti-gelsolin antibodies (St Louis, USA), respectively (final volume 30 μl). Protein G–Sepharose (Pharmacia) was added and after 1 h incubation at room temperature the sample was centrifuged for 2 min (500 *g*). Pellets were washed three times with 1 ml TDA buffer (10 mM Tris–HCl pH 7.5, 1 mM dithiothreitol, 0.02% NaN_3) supplemented with 150 mM NaCl, 1% Triton X-100 and 0.5% NP-40 (for fragminP–actin) or G-buffer (for actin–gelsolin) with the same amounts of NaCl, Triton X-100 and NP-40, followed by three washes with TDA or G-buffer without NaCl/Triton X-100/NP-40. The immunoprecipitates were boiled for 2 min in Laemmli sample buffer (Laemmli, 1970), followed by SDS–PAGE gel electrophoresis on 10% mini-slab gels according to Matsudaira and Burgess (1978).

Immunoblotting on nitrocellulose membranes was performed as described by Towbin *et al*. (1979). The membranes were quenched in Tris-buffered saline (TBS) (10 mM Tris–HCl pH 7.4, 140 mM NaCl) containing 4% skimmed milk powder for 3 h at room temperature. Primary antibody was added and incubated for 3 h in TBS/1% milk powder. After three washes in TBS/1% milk powder the secondary antibody (goat anti-rabbit antibody coupled to alkaline phosphatase) was added for 3 h at room temperature. Proteins were visualized in the developing buffer (100 mM $NaHCO_3$ pH 9.8, 2 mM $MgCl_2$) containing NBT (nitro-blue tetrazolium chloride) and BCIP (bromo-chloro-indolyl phosphate) (purchased from Duchefa, Haarlem, The Netherlands).

Quantitative measurement of actin dissociation from the fragminP–actin complex by (lysophospho)lipids

Native fragminP–actin was quantitatively phosphorylated (De Corte *et al*., 1996) with recombinant actin–fragmin kinase, purified according to Eichinger *et al*. (1996). Immunoprecipitation of phosphorylated fragminP–actin following incubation with various lipids was performed as described above. The supernatants were counted in a Wallac 1409 liquid scintillation counter (Pharmacia/LKB) or the pellets were analyzed by SDS–PAGE and Coomassie Blue staining. FragminP–actin bands were excised and radioactivity was measured.

Phosphorylation of actin-binding proteins by $pp60^{c\text{-}src}$

Phosphorylation of gelsolin and fragminP by $pp60^{c\text{-}src}$ was performed as described previously (De Corte *et al*., 1997). The proteins were preincubated with LPA or PI 4,5-P_2 for 30 min at room temperature. Reactions were terminated by the addition of 5× concentrated Laemmli sample buffer and analyzed by SDS–PAGE on mini-slab gels. Radioactivity was detected by autoradiography and quantified by scanning the autoradiograms with a 2202 Ultroscanner (Pharmacia/LKB) or by Cerenkov counting of the excised gel bands.

Preparation of platelet suspensions

Platelets were acquired from the local blood bank and prepared according to Ardlie *et al*. (1970). Briefly, platelets were sedimented by centrifugation at 1100 *g* for 20 min and then resuspended in modified Tyrode's solution I, pH 6.5 (without calcium), containing 2 mM magnesium and 0.2 mM EGTA. (Tyrode's solution contains 2 mM $CaCl_2$, 2.5 mM KCl, 0.5 mM MgCl, 150 mM NaCl, 0.35 mM $NaH_2PO_4.H_2O$ and 5 mM D-glucose.) Cells were again pelleted by centrifugation at 1100 *g* for 20 min and resuspended in Tyrode's solution II (Tyrode's solution I without EGTA). After pelleting, the platelets were finally resuspended in Tyrode's solution III, pH 7.35.

Release of gelsolin from permeabilized resting platelets

Lipid-mediated release of gelsolin from permeabilized platelets was studied essentially as described in Barkalow *et al*. (1996) and Hartwig *et al*. (1995). Briefly, platelets (1.4×10^{12}/ml) were permeabilized for 30 s with 0.4% octylglucoside in 1/10 volume 10× PHEM buffer (0.6 M PIPES, 0.25 M HEPES, 0.1 M EGTA, 2 mM $MaCl_2$, pH 6.9), protease inhibitors (10 μg/ml aprotenin, 10 μg/ml leupeptin, 1 mM PMSF), 0.1 μM phallacidin, and centrifuged for 1 min at 10 000 *g*. The pellet was washed 5 times by resuspending in 1× PHEM buffer, 0.1 μM pallacidin and protease inhibitors. The pellet was finally resuspended in PHEM buffer and divided into equal aliquots. Buffer (control) or lipids were added to the suspensions and incubated for 5 min. The sample was then centrifuged for 1 min at 10 000 *g* and the supernatant was recovered. Samples were taken at each step for SDS–PAGE analysis and Western blotting.

Quenching of intrinsic tryptophane fluorescence

Fluorescence emission spectra were recorded at room temperature using an SFM25 fluorimeter (Kontron Instruments, Zürich). The tryptophane fluorescence spectrum of a 1 μM fragminP solution in TDA buffer (pH 7.5) was recorded by excitation at 292 nm. LPA or PIP_2 were added at 1 μl increments, and the fluorescence spectra were recorded 5 min after each addition. The total volume of added lipids did not exceed 2% of the initial fragminP solution volume. The decrease in fluorescence emission at 320 nm was plotted as a function of lipid concentration and the apparent dissociation constant was calculated using the GraphPad Prism program (Graphpad Software).

Mass spectrometry

To ascertain the purity of LPA and PI 4,5-P_2, aliquots were subjected to electrospray–ionization mass spectrometry (ESI-MS). The instrument consists of a single quadrupole mass analyzer VG platform (VG Biotech Fisons Instruments, Manchester, UK). Approximately 100 pmol was introduced into the ion source with a HPLC pump (Pharmacia LKB-HPLC pump 2248) at a flow rate of 5 μl/min. Scans were made for 6 s between m/z 300 and m/z 1300 in the negative ionization mode.

Miscellaneous

All protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. The villin 133-YNVQRLLHVKGKKNVV-147 (Arpin *et al*., 1988; Gettemans *et al*., 1995) PI 4,5-P_2-binding peptide was synthesized on an Applied Biosystems automated 431A Peptide Synthesizer.

Acknowledgements

We thank Dr E.Friederich (Institut Curie, Paris) for the generous gift of gelsolin cDNA and Dr J.Robbens (University of Gent) for adseverin cDNA. This work was supported by grants from the Flanders Institute for Science and Technology (VLAB-COT), the Concerted Research Actions of the Flemish Community (GOA), the Fund for Scientific Research (F.W.O.) and the EU contract CHRX-CT94-0652 to J.V. J.G. is a Postdoctoral Fellow of the Fund for Scientific Research -Flanders (Belgium) (F.W.O.).

References

Ahmed,S., Lee,J., Kozma,R., Best,A., Monfires,C. and Lim,L. (1993) A novel functional target for tumour-promoting phorbol esters and lysophosphatidic acid. *J. Biol. Chem.*, **268**, 10709–10712.

An,S., Avery Dickens,M., Bleu,T., Hallmark,O.G. and Goetzl,E.J. (1997) Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem. Biophys Res. Commun*., **231**, 619–622.

André,E., Lottspeich,F., Schleicher,M. and Noegel,A. (1988) Severin, gelsolin and villin share a homologous sequence in regions presumed to contain F-actin severing domains. *J. Biol. Chem.*, **263**, 722–727.

Arcaro,A. (1998) The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. *J. Biol. Chem.*, **273**, 805–813.

Ardlie,N.G., Packham,M.A. and Mustard,J.F. (1970) Adenosine diphosphate-induced platelet aggregation in suspensions of washed rabbit platelets. *Br. J. Haematol*., **19**, 7–17.

Arpin,M., Pringault,E., Finidori,J., Garcia,A., Jeltsch,J.-M., Vandekerckhove,J. and Louvard,D. (1988) Sequence of human villin: a large duplicated domain homologous with other actin-severing proteins and a unique small carboxy-terminal domain related to villin specificity. *J. Cell Biol.*, **107**, 1759–1766.

Barkalow,K., Witke,W., Kwiatkowski,D.J. and Hartwig,J.H. (1996) Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. *J. Cell Biol.*, **134**, 389–399.

Bengtsson,T., Rundquist,I., Stendahl,O., Wymann,M.P. and Andersson,T. (1988) Increased breakdown of phosphatidylinositol, 4,5-bisphosphate is not an initiating factor for actin assembly in human neutrophils. *J. Biol. Chem.*, **263**, 17385–17389.

Benton,A.M., Gerrard,J.M., Michiel,T. and Kindom,S.E. (1982) Are lysophosphatidic acids or phosphatidic acids involved in stimulus activation coupling in platelets? *Blood*, **60**, 642–649.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Brenner,S.L. and Korn,E.D. (1983) On the mechanism of actin monomer–polymer subunit exchange at steady state. *J. Biol. Chem.*, **258**, 5013–5020.

Chellaiah,M. and Hruska,K. (1996) Osteopontin stimulates gelsolin-associated phosphoinositide levels and phosphatidylinositol triphosphate-hydroxyl kinase. *Mol. Biol. Cell*, **7**, 743–753.

Chettibi,S., Lawrence,A.J., Stevenson,R.D. and Young,J.D. (1994) Effect of lysophosphatidic acid on motility, polarisation and metabolic burst of human neutrophils. *FEMS Immunol. Med. Microbiol.*, **8**, 271–282.

Cooper,J.A., Bryan,J., Schwab III,B., Frieden,C., Loftus,D.J. and Elson,E.L. (1987) Microinjection of gelsolin into living cells. *J. Cell Biol.*, **104**, 491–501.

Cuvillier,O., Rosenthal,D.S., Smulson,M.E. and Spiegel,S. (1998) Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J. Biol. Chem.*, **273**, 2910–2916.

Dadabay,C.Y., Patton,E., Cooper,J.A. and Pike,L.J. (1991) Lack of correlation between changes in polyphosphoinositide levels and actin/gelsolin complexes in A431 cells treated with epidermal growth factor. *J. Cell Biol.*, **112**, 1151–1156.

Das,A.K. and Hajra,A.K. (1992) Critical micellar concentrations of palmitoyl dihydroxyacetone phosphate and 1-palmitoyl-rac-glycerol 3-phosphate. *J. Biol. Chem.*, **267**, 9731 (Appendix).

De Corte,V., Gettemans,J., Waelkens,E. and Vandekerckhove,J. (1996) *In vivo* phosphorylation of actin in Physarum polycephalum. Study of the substrate specificity of the actin-fragmin kinase. *Eur. J. Biochem.*, **241**, 901–908.

De Corte,V., Gettemans,J. and Vandekerckhove,J. (1997) Phosphatidylinositol, 4,5-bisphosphate specifically stimulates $pp60^{c\text{-}src}$ catalyzed phosphorylation of gelsolin and related actin-binding proteins. *FEBS Lett.*, **401**, 191–196.

Eberle,M., Traynor-Kaplan,A.E., Sklar,L.A. and Norgauer,J. (1990) Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? *J. Biol. Chem.*, **265**, 16725–16728.

Eichholtz,T., Jalink,K., Fahrenfort,I. and Moolenaar,W.H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.*, **291**, 677–680.

Eichinger,L., Bomblies,L., Vandekerckhove,J., Schleicher,M. and Gettemans,J. (1996) A novel type of protein kinase phosphorylates actin in the actin–fragmin complex. *EMBO J.*, **15**, 5547–5556.

Fourcade,O., Simon,M.-F., Viodé,C., Rugani,N., Leballe,F., Ragab,A., Fournié,B., Sarda,L. and Chap,H. (1995) Secretory phospholipiase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell*, **80**, 919–927.

Fukami,K. and Takenawa,T. (1992) Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balb/c 3T3 cells is a potent mitogenic signal. *J. Biol. Chem.*, **267**, 10988–10993.

Gettemans,J., De Ville,Y., Vandekerckhove,J. and Waelkens,E. (1992) Physarum actin is phosphorylated as the actin–fragmin complex at residues Thr203 and Thr202 by a specific 80 kDa kinase. *EMBO J.*, **11**, 3185–3191.

Gettemans,J., De Ville,Y., Vandekerckhove,J. and Waelkens,E. (1993) Purification and partial amino acid sequence of the actin–fragmin kinase from Physarum polycephalum. *Eur. J. Biochem.*, **214**, 111–119.

Gettemans,J., De Ville,Y., Waelkens,E. and Vandekerckhove,J. (1995) The actin-binding properties of the Physarum actin–fragmin complex. Regulation by calcium, phospholipids and phosphorylation. *J. Biol. Chem.*, **270**, 2644–2651.

Goldschmidt-Clermont,P.J., Machesky,L.M., Baldassare,J.J. and Pollard,T.D. (1990) The actin-binding protein profilin binds to PI, 4,5-P2 and inhibits its hydrolysis by phospholipase C. *Science*, **247**, 1575–1578.

Guo,Z., Liliom,K., Fisher,D.J., Bathurst,I.C., Tomei,L.D., Kiefer,M.C. and Tigyi,G. (1996) Molecular cloning of a high-affinity receptor for the growth factor-like lipid mediator lysophosphatidic acid from *Xenopus* oocytes. *Proc. Natl Acad. Sci. USA*, **93**, 14367–14372.

Hartwig,J.H., Bokoch,G.M., Carpenter,C.L., Janmey,P.A., Taylor,L.A., Toker,A. and Stossel,T.P. (1995) Thrombin receptor ligation and activated rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell*, **82**, 643–65.

Hecht,J.H., Weiner,J.A., Post,S.R. and Chun,J. (1996) Ventricular zone gene 1 (Vzg 1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell Biol.*, **135**, 1071–1083.

Isenberg,G. and Goldman,W.H. (1995) Actin-binding proteins–lipid interactions. *Cytoskeleton*, **1**, 169–204.

Jalink,K., van Corven,E.J. and Moolenaar,W.H. (1990) Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+}-mobilizing stimulus for fibroblasts. *J. Biol. Chem.*, **265**, 12232–12239.

Janmey,P.A. (1994) Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.*, **56**, 169–191.

Janmey,P.A. and Stossel,T.P. (1987) Modulation of gelsolin function by phosphatidylinositol, 4,5-bisphosphate. *Nature*, **325**, 362–364.

Janmey,P.A., Iida,K., Yin,H.L. and Stossel,T.P. (1987) Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin–actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. *J. Biol. Chem.*, **262**, 12228–122236.

Janmey,P.A., Lamb,J., Allen,P.G. and Matsudaira,P.T. (1992) Phosphoinositide-binding peptides derived from the sequences of gelsolin and villin. *J. Biol. Chem.*, **267**, 11818–11823.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lauener,R., Shen,Y., Duronio,V. and Salari,H. (1995) Selective inhibition of phosphatidylinositol 3-kinase by phosphatidic acid and related lipids. *Biochem. Biophys Res. Comm.*, **215**, 8–14.

Lee,M.-J., Van Brocklyn,J.R., Thangada,S., Liu,C.H., Hand,A.R., Menzeleev,R., Spiegel,S. and Hla,T. (1998) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science*, **279**, 1552–1555.

Lin,K.-M., Wenegieme,E., Lu,P.-J., Chen,C.-S. and Yin,H.L. (1997) Gelsolin binding to phosphatidylinositol, 4,5-bisphosphate is modulated by calcium and pH. *J. Biol. Chem.*, **272**, 20443–20450.

Low,M.G. and Huang,K.-S. (1993) Phosphatidic acid, lysophosphatidic acid and lipid A are inhibitors of glycosylphosphatidylinositol-specific phospholipase D. *J. Biol. Chem.*, **268**, 8480–8490.

Lu,P.-J., Shieh,W.-R., Rhee,S.G., Yin,H.L. and Chen,C.-S. (1996) Lipid products of phosphinositide 3-kinase bind human profilin with high affinity. *Biochemistry*, **35**, 14027–14034.

Matsudaira,P. and Burgess,D.R. (1978) SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.*, **87**, 386–396.

Meyer zu Heringdorf,D. *et al.* (1998) Sphingosine kinase-mediated Ca^{2+} signalling by G-protein-coupled receptors. *EMBO J.*, **17**, 2830–2837.

Moolenaar,W.H. (1995) Lysophosphatidic acid, a multifunctional phospholipid messenger. *J. Biol. Chem.*, **22**, 12949–12952.

Moolenaar,W.H., Kranenburg,O., Postma,F.R. and Zondag,G.C. (1997) Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr. Opin. Cell Biol.*, **9**, 168–173.

Olivera,A. and Spiegel,S. (1993) Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature*, **365**, 557–560.

Peppelenbosch,M.P, Tertoolen,L.G.J., den Hertog,J. and de Laat,S.W. (1992) Epidermal growth factor activates calcium channels by phospholipase A_2/5-lipoxygenase-mediated leukotriene C_4 production. *Cell*, **69**, 295–303.

Rani,C.S.S. *et al.* (1997) Divergence in signal transduction pathways of platelet derived growth factor (PDGF) and epidermal growth factor (EGF) receptors: involvement of sphingosine-1-phosphate in PDGF but not EGF. *J. Biol. Chem.*, **272**, 10777–10783.

Robbens,J., Louahed,J., De Pestel,K., Vancolen,I., Ampe,C., Vandekerckhove,J. and Renauld,J.-C. (1998) Murine adseverin (D5), a novel member of the gelsolin family and murine adseverin are induced by interleukin-9 in T-helper lymphocytes. *Mol. Cell. Biol.*, **18**, 4589–4596.

Sando,J.J. and Chertihin,O.I. (1996) Activation of protein kinase C by lysophosphatidic acid: dependence on composition of phospholipid vesicles. *Biochem. J.*, **317**, 583–588.

Singh,S.S., Chauhan,A., Murakami,N. and Chauhan,V.P.S. (1996) Profilin and gelsolin stimulate phosphatidylinositol 3-kinase activity. *Biochemistry*, **35**, 16544–16549.

Spudich,J.A. and Watt,S. (1971) The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.*, **246**, 4866–4871.

Stossel,T.P. (1993) On the crawling of animal cells. *Science*, **260**, 1086–1094.

Svetlov,S. and Nigam,S. (1993) Evidence for the presence of specific high affinity cytosolic binding sites for platelet-activating factor in human neutrophils. *Biochem. Biophys. Res. Commun.*, **190**, 162–166.

T'Jampens,D. *et al*. (1997) Molecular cloning, over-expression, developmental regulation and immunolocalization of fragminP, a gelsolin-related actin-binding protein from Physarum polycephalum microplasmodia. *J. Cell Sci.*, **110**, 1215–1226.

Toker,A. and Cantley,L.C. (1997) Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature*, **387**, 673–676.

Towbin,H., Staehelin,T. and Gordon,J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.

Tsai,M.-H., Hall,A. and Stacey,D.W. (1989) Inhibition by phospholipids of the interaction between R-ras, rho and their GTP-ase activating proteins. *Mol. Cell. Biol.*, **9**, 5260–5264.

Van Brocklyn,J.R. *et al*. (1998) Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J. Cell Biol.*, **142**, 229–240.

Wang,F., Nobes,C.D., Hall,A. and Spiegel,S. (1997) Sphingosine-1-phosphate stimulates Rho-mediated tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 fibroblasts. *Biochem. J.*, **324**, 481–488.

Watson,S.P., McConnell,R.T. and Lapetina,E.G. (1985) Decanoyl lysophosphatidic acid induces platelet aggregation through an extracellular action. Evidence against a second messenger role for lysophosphatidic acid. *Biochem. J.*, **232**, 61–66.

Yamamura,S., Sadahira,Y., Ruan,F., Hakomori,S. and Igarashi,Y. (1996) Sphingosine-1-phosphate inhibits actin nucleation and pseudopodium formation to control cell motility of mouse melanoma cells. *FEBS Lett.*, **382**, 193–197.

Yatomi,Y., Yamamura,S., Ruan,F. and Igarashi,Y. (1997) Sphingosine 1 phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.*, **272**, 5291–5297.

Yu,F.X., Johnston,P.A., Südhof,T.C. and Yin,H.L. (1990) gCap39, a calcium-ion and polyphosphoinositide-regulated actin capping protein. *Science*, **250**, 1413–1415.

Yu,F.X., Sun,H.Q., Janmey,P.A. and Yin,H.L. (1992) Identification of a phosphoinositide-binding sequence in an actin monomer-binding domain of gelsolin. *J. Biol. Chem.*, **267**, 14616–14621.

Zhang,H., Desai,N.N., Olivera,A., Seki,T., Brooker,G. and Spiegel,S. (1995) Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell Biol.*, **114**, 155–167.

Received July 20, 1998; revised August 26, 1998; accepted August 27, 1998