Phosphatidylinositol 4,5-bisphosphate specifically stimulates PP60^{c-src} catalyzed phosphorylation of gelsolin and related actin-binding proteins

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Abstract Gelsolin is a widely distributed Ca^{2+}-dependent regulator of the cortical actin network. We demonstrate that gelsolin is phosphorylated by pp60^{c-src} and that this phosphorylation is dramatically enhanced by phosphatidylinositol 4,5-bisphosphate (PIP_2), known to specifically interact with gelsolin. Other phospholipids display only a marginal effect. pp56^{lck}, a tyrosine kinase of the same family, does not phosphorylate gelsolin. Other mammalian actin-binding proteins such as profilin and CapG but also fragmin from *Physarum polycephalum* are similar targets for PIP_2-stimulated pp60^{c-src} phosphorylation.

Key words: Gelsolin; Phosphorylation; pp60^{c-src}; Protein kinase C; Phosphatidylinositol 4,5-bisphosphate

1. Introduction

pp60^{c-src} is the normal cellular counterpart of the transforming protein of Rous sarcoma virus (RSV) pp60^{v-src} and is a membrane-associated non-receptor tyrosine kinase (for reviews see [1,2]). This kinase plays an important role in the control of cell division [3], cell growth and differentiation [4] and has a distinctive function in bone formation [5]. Significant amounts of pp60^{c-src} are present in blood platelets [6] and neurons [7] suggesting that the role of pp60^{c-src} is not only restricted to growth and differentiation of proliferating cells but may display other functions.

Next to autophosphorylation [8] resulting in its activation, pp60^{c-src} is known to phosphorylate a number of other target proteins including pp125 focal adhesion kinase [9], p80/85 cortactin [10,11], an actin filament-binding protein [12], the ras GTPase activating protein [13] and possibly also the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI-3 kinase) [14].

Gelsolin is a actin-binding protein (82 kDa) present in the cytoplasm of vertebrate cells and blood plasma [15,16]. The secreted form is structurally similar to the cytoplasmic isoform but has an additional leader sequence of 25 amino acids. Both proteins are encoded by a single gene and the isoforms have the same functional properties. In vitro, gelsolin severs actin filaments, caps the fast-growing end and promotes growth of actin filaments by creating nucleation sites [15,17,18]. These activities are enhanced by Ca^{2+} whereas polyphosphoinositides such as PIP_2 and PIP are known to act as inhibitors, in particular of the severing activity (reviewed in [19]). Polyphosphoinositides can also uncap gelsolin from the fast-growing end of actin filaments in vivo, and thus indirectly promote actin filament growth [20].

Several members of the gelsolin family are phosphorylated in vitro and/or in vivo by different protein kinases. For instance, CapG, a strictly actin-capping protein, is phosphorylated in macrophages on serine and threonine residues but neither the corresponding kinase nor the sites have yet been identified [21]. Also profilin, a ubiquitously expressed small actin monomer binding protein was reported to be phosphorylated in vitro by protein kinase C (PKC). Interestingly, PIP_2 stimulated this activity by modifying the nature of the profilin–PKC interaction [22]. Fragmin, a Ca^{2+}-dependent actin-binding protein from *Physarum polycephalum* displaying properties similar to gelsolin, is phosphorylated by two variants of an endogenous casein kinase II [23].

It has been stated previously that gelsolin is neither phosphorylated in macrophages [21] nor in Rous sarcoma virus-transformed rat cells [24], although indirect evidence supports the opposite since recently Chellaiah and Hruska [25] suggested that in osteoclasts pp60^{c-src} and gelsolin could interact transiently with each other. Osteopontin was found to stimulate the association of phosphoinositides with gelsolin through stimulation of the PI-3 kinase. It was therefore postulated that pp60^{c-src} could be the linker molecule between gelsolin and PI-3 kinase [25].

In the present study we have investigated phoshorylation of gelsolin by pp60^{c-src}. We show that phospholipids, in particular PIP_2 and PIP, dramatically enhance the tyrosine-specific phosphorylation of gelsolin. This finding suggests a new regulatory role for PIP_2. pp56^{lck}, another member of the src-kinase family, did not phosphorylate gelsolin, illustrating the specificity of pp60^{c-src} for gelsolin. We further show that the effect of PIP_2 is not restricted to gelsolin alone but also applies to other actin-binding proteins such as profilin, fragmin and CapG. The results are discussed in terms of a possible direct role of pp60^{c-src} in the organization of the actin meshwork associated with the plasma membrane of eukaryotic cells.

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Abbreviations: Cer, ceramide; DTT, dithiothreitol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI-3 kinase, phosphatidylinositol-3 kinase; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

2. Materials and methods

2.1. Materials

Nitrocellulose was obtained from Amersham (Buckinghamshire, UK). Whatman 3MM paper was from Whatman (Maidstone, UK). Cyanogen bromide activated Sepharose beads were purchased from Pharmacia/LKB (Uppsala, Sweden). DNase I was from Worthington biochemical corporation (New Jersey, USA). [γ-^{32}P]ATP (4500 Ci/

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mmol) was from ICN Biochemicals (Costa Mesa, USA). Poly(L-proline) (average M_r 10000–30000 Da) was from Sigma (St. Louis, MO). Cellulose thin-layer plates were obtained from Merck (Darmstadt, Germany).

2.2. Proteins

Gelsolin and CapG were purified from human plasma according to Cooper et al. [26] and Johnston et al. [27], respectively. The experiments were performed with plasma gelsolin; some experiments (indicated in the text) were repeated with cytoplasmic gelsolin that was purified as described by Bryan [28]. Recombinant fragmin and platelet profilin were purified as described by T'Jampens et al. (unpublished results) and by Lind et al. [29], respectively. Monoclonal anti-gelsolin antibodies were obtained from Sigma (St. Louis, MO). Biotin-conjugated 4G10 anti-phosphotyrosine antibody was obtained from Upstate Biotechnology Incorporated (UBI, New York, NY). pp60^{c-src} (recombinant human src kinase) with a specific activity of 900000 U/mg kinase transferred 2 pmol/min per unit to the src kinase substrate peptide. pp56^{lck} (partially purified from membrane fractions of bovine thymus) with a specific activity of 6406 U/mg transferred more than 1 pmol phosphate/min per mg kinase to src kinase substrate peptide. PKC (a mixture of α-, β- and γ-isoforms) was isolated from rat brain and transferred 22.86 μmol ^{32}P/min per mg enzyme to the PKC substrate peptide. The three kinases were all obtained from UBI.

2.3. Preparation of lipids

Phospholipids that were obtained as a lyophilized salt were dissolved in water at the indicated concentrations (PIP_2, 5 mM; PIP, 1 mM; PI, 1 mM; PA, 5 mM and PS, 1 mM). Other phospholipids obtained as chloroform solutions (PC, PE) were dried in a stream of nitrogen and dissolved in 10 mM Tris-HCl, pH 8.0, at a concentration of 5 mM. Ceramide was dissolved in ethanol at 10 mM. All the lipids were sonicated for 30 min in a Branson S75 sonicator (Branson Sonic Power Co., Danbury, CT) and stored in aliquots at −80°C. PC, PE and to a lesser extent PS remained turbid after sonication. Before use the lipids were sonicated for an additional 5 min.

2.4. Phosphorylation assays

Phosphorylation of gelsolin was carried out at 30°C for the indicated periods of time with or without PIP_2 in a total volume of 12.5 μl containing 2.5 μM gelsolin, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (specific radioactivity 1000–1500 cpm/pmol) and 2.5 U pp60^{c-src} or 2.5 U pp56^{lck} in 10 mM Tris-HCl, pH 7.5, and 1 mM DTT. Gelsolin was preincubated with PIP_2 for 30 min at RT with 30-, 60- or 90-fold molar excess over gelsolin, corresponding with 75 μM, 150 μM and 225 μM, respectively. Phosphorylation of gelsolin (2.5 μM) with PKC (15 ng) was performed in the presence of 80 μg/ml phosphatidylserine and 0.4 mM Ca^{2+}. Reactions were terminated by the addition of 5× concentrated Laemmli sample buffer [30] and analyzed by 1D SDS-polyacrylamide mini-slab gel electrophoresis [31]. The radioactivity incorporated into gelsolin was detected by autoradiography and quantified by scanning the autoradiograms with a 2202 Ultroscanner (Pharmacia/LKB) or by Čerenkov counting of the exised gel bands using a Wallac 1409 scintillation counter (Pharmacia/LKB).

Phosphorylation of fragmin (2.5 μM), CapG (2.5 μM) and profilin (7 μM) with pp60^{c-src} (2.5 U) was carried out as described for gelsolin.

2.5. Phospho-amino acid analysis

Gelsolin (2 μM), preincubated with PIP_2 (30-fold molar excess relative to gelsolin) was phosphorylated for 1 h with PKC (150 ng) or pp60^{c-src} (30 U) in a total volume of 60 μl. Phosphogelsolin was purified by reversed-phase high performance liquid chromatography through a C_{18} column (4.6×250 mm, 5 mM particle size, 300 Å pore size, Vydac, Separations Group, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid (TFA) and proteins were eluted with a linear gradient of 0–90% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The gradient was started 5 min after sample loading and terminated after 70 min. Phosphorylated gelsolin eluted after 45 min and was dried in a Speed-Vac (Savant Instruments, Farmingdale, NY) and hydrolyzed with 6 N HCl for 2 h at 110°C. One-dimensional cellulose thin-layer chromatography was performed as described by Ejiri [32]. Radioactive amino acids were detected by autoradiography and the unlabeled standards were stained with ninhydrin.

2.6. Miscellaneous

Protein concentrations were determined by the method of Bradford [33] using bovine serum albumin as standard.

The gelsolin peptide 160-QRLFQVKGRR-169 was synthesized with a 431A peptide synthesizer (Applied Biosystems).

Western blot analysis was performed as described by Towbin et al. [34]. The biotin-conjugated anti-phosphotyrosine monoclonal antibody was used at a concentration of 1 μg/ml and incubated with gelsolin overnight at 4°C. The secondary streptavidin-conjugated alkaline phosphatase (Sigma) was diluted 1:2000 and incubated with the primary immunecomplex for 2 h at room temperature.

3. Results

3.1. Phosphorylation of gelsolin by pp60^{c-src} is stimulated by PIP_2

In an earlier report it was mentioned that fragmin, an actin-binding protein of the gelsolin family and isolated from *P. polycephalum*, was phosphorylated by pp60^{c-src} [35]. The tyrosine residue in the 114-LDDYL-118 motif was postulated as a candidate target [36] in view of its conformity with pp60^{src} consensus phosphorylation sites [37] and because this conserved region was predicted to be an actin-binding site [38]. Based on more recent X-ray crystallographic data from the S_1-gelsolin-actin complex [39], the importance in actin binding of this motif was confirmed. This raised the attractive hypothesis of regulation of actin binding through pp60^{c-src} phosphorylation. These observations and a more recent report alluding to an association between gelsolin and pp60^{c-src} [25], prompted us to investigate in more detail the possible phosphorylation of gelsolin by pp60^{c-src}.

When gelsolin was incubated with recombinant pp60^{c-src} and Mg^{2+}/[γ-^{32}P]ATP we noticed a time dependent phospho-

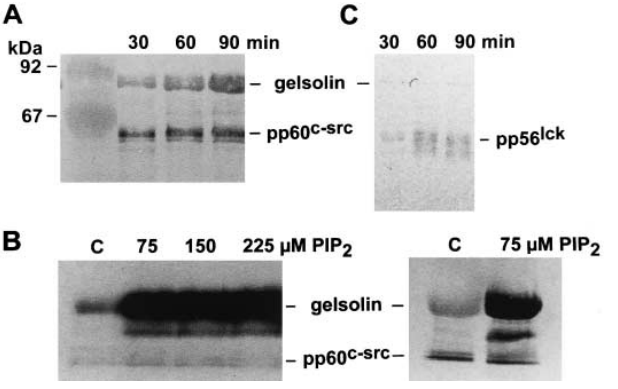

Fig. 1. Phosphorylation of gelsolin by pp60^{c-src} and stimulation by PIP_2. A: Western blot analysis of gelsolin phosphorylation by pp60^{c-src} in the absence of PIP_2. Gelsolin (2.5 μM) was phosphorylated for the indicated time intervals with 2.5 U recombinant pp60^{c-src} and Western blotted with anti-phosphotyrosine antibodies. pp60^{c-src} is visible as an autophosphorylated band of 60 kDa. B: Stimulation of gelsolin phosphorylation by PIP_2. Gelsolin (2.5 μM) was preincubated with the indicated concentrations of PIP_2 for 30 min at room temperature and then phosphorylated with 2.5 U of pp60^{c-src} for 10 min. The phosphorylation mixture was analyzed by SDS-PAGE and autoradiography (overnight exposure at −80°C). c: control, gelsolin phosphorylation in the same conditions as above but in the absence of PIP_2 (left panel). Anti-phosphotyrosine Western blot of gelsolin phosphorylated by pp60^{c-src} in the absence and presence of 75 μM PIP_2 (right panel). C. pp56^{lck} does not phosphorylate gelsolin. Experimental conditions were the same as in A. Note the autophosphorylation of pp56^{lck} as well as two other tyrosine phosphorylated proteins present in the partially purified pp56^{lck} preparation.

rylation of gelsolin (Fig. 1A). A slower rate of phosphorylation was observed when Mn^{2+} was substituted for Mg^{2+} (data not shown). Western blot analyses with monoclonal anti-phosphotyrosine antibodies confirmed that gelsolin was phosphorylated on tyrosine residue(s) (Fig. 1A).

PIP_2 is known to associate tightly with gelsolin in vitro and in vivo [40,20], inhibiting gelsolin's F-actin-severing activity [41]. Interestingly, Hansson et al. [22] demonstrated that PIP_2 stimulates profilin phosphorylation by PKC. We therefore analyzed the effect of this phospholipid on $pp60^{c-src}$-mediated phosphorylation of plasma gelsolin or cytoplasmic gelsolin after preincubation with various concentrations of PIP_2. A very strong stimulation of gelsolin phosphorylation was noticed (Fig. 1B). A 30-fold molar excess of PIP_2 (75 μM) relative to gelsolin resulted in a 25-fold increase of gelsolin phosphorylation by $pp60^{c-src}$. This was measured after 10 min phosphorylation by densitometric scanning of the corresponding autoradiogram or by counting the radiolabel. The corresponding phospho-amino acid analysis is shown in Fig. 5B. The degree of phosphorylation further increased by 40% when the reaction was allowed to proceed for 90 min. Higher concentrations of PIP_2 (225 μM; a 90-fold molar excess) produced an additional increase of ^{32}P-incorporation, resulting in up to 30-fold increase of gelsolin phosphorylation by $pp60^{c-src}$ measured after 10 min (Fig. 1B).

In the absence of PIP_2 we measured an incorporation of 0.007 mol phosphate/mol of gelsolin. However, in the presence of a 30-fold or 90-fold molar excess of PIP_2, stoichiometries of 0.12 mol phosphate/mol of gelsolin and 0.18 mol phosphate/mol of gelsolin were observed, respectively. Using a reciprocal Michaelis-Menten plot, we derived a K_m value of ~6 μM.

3.2. *$pp56^{lck}$ does not phosphorylate gelsolin*

The 56 kDa T lymphocyte-specific tyrosine kinase $pp56^{lck}$ is a member of the src family and has been implicated in T-cell maturation and activation [42,43]. To determine the specificity of tyrosine kinases able to phosphorylate gelsolin, we studied the phosphorylation of gelsolin by $pp56^{lck}$. $pp56^{lck}$, obtained as a partially purified preparation from membranes of bovine thymus, was not able to phosphorylate gelsolin on tyrosine as demonstrated by Western blot analysis (see Fig. 1C). Even in the presence of PIP_2 no phosphorylation was observed. Tyrosine phosphorylation activity by $pp56^{lck}$ was evidenced by efficient phosphorylation of poly (Glu/Tyr) 4:1, a well-known substrate for tyrosine kinases [44] (data not shown).

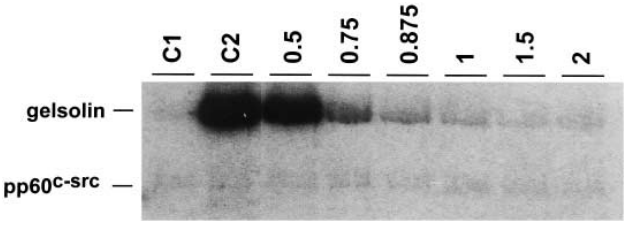

Fig. 2. PIP_2 enhances phosphorylation through association with gelsolin. PIP_2 (75 μM) was preincubated with various concentrations of the PIP_2-binding gelsolin-peptide (160-QRLFQVKGRR-169) and subsequently added to 2.5 μM gelsolin and $pp60^{c-src}$. Molar ratios of gelsolin/peptide: PIP_2 are indicated on top. c_1: $control_1$, gelsolin (2.5 μM) phosphorylated with $pp60^{c-src}$ for 10 min without preincubation of PIP_2. c_2: $control_2$, gelsolin (2.5 μM) preincubated with a 30-fold molar excess of PIP_2 (75 μM) and subsequently phosphorylated with $pp60^{c-src}$. The autoradiogram was exposed for 7 h at −80°C.

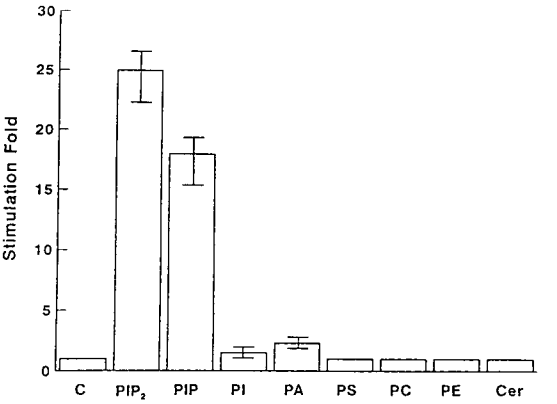

Fig. 3. Specificity of PIP_2 in stimulating the phosphorylation of gelsolin by $pp60^{c-src}$. (Phospho)lipids (75 μM each) are shown on the *X*-axis and were preincubated with gelsolin (2.5 μM) for 30 min before $pp60^{c-src}$ was added. The stimulation of phosphorylation relative to the control (without PIP_2) is represented on the *Y*-axis. The results are representative for three independent experiments.

3.3. *PIP_2 does not activate $pp60^{c-src}$ kinase activity but acts on the substrate*

Although PIP_2 is known to interact with gelsolin and other actin-binding proteins [40,45,46], it cannot be excluded at this stage that the phospholipid acts as a stimulator for the kinase. For example, PIP_2 has been shown to function as a cofactor for PLD [47] or as an enhancer for G-protein-coupled receptor kinase activity [48]. To determine the mode of action of PIP_2 in the phosphorylation of gelsolin, we phosphorylated gelsolin with $pp60^{c-src}$ after preincubation of PIP_2 with a phosphoinositide-binding peptide derived from gelsolin. Various amounts of the PIP_2-binding gelsolin peptide, 160-QRLFQVKGRR-169 [49] were preincubated with PIP_2 (75 μM) and subsequently gelsolin was added. Upon addition of increasing concentrations of the PIP_2-binding peptide, the phosphorylation of gelsolin was increasingly inhibited. At a 1:1 molar ratio of peptide/PIP_2, the stimulation of gelsolin phosphorylation was nearly completely abolished (Fig. 2). A 1.5 molar excess of peptide over PIP_2 reduced the phosphorylation by $pp60^{c-src}$ to control levels (Fig. 2). These results strongly suggest that PIP_2 binds to gelsolin and converts it into a good substrate for $pp60^{c-src}$.

To eliminate the possibility that PIP_2 could also activate $pp60^{c-src}$ under our experimental conditions, we used RCM-lysozyme as an alternative substrate [44]. We observed no variations in the rate of tyrosine phosphorylation of the latter when PIP_2 was added in the same concentrations as mentioned above (data not shown). Taken together, the lack of stimulation of $pp60^{c-src}$ activity by PIP_2 and the efficient competition between gelsolin and a PIP_2-binding synthetic peptide derived from gelsolin indicate that the dramatically increased rate of phosphorylation is due solely to the PIP_2–gelsolin interaction.

3.4. *Phospholipid-specificity in the stimulation of gelsolin phosphorylation*

To study the structural requirements that are necessary for a phospholipid to stimulate phosphorylation of gelsolin, we

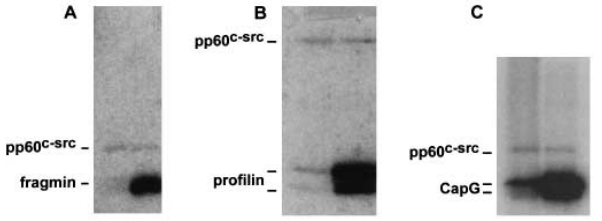

Fig. 4. Phosphorylation of fragmin, profilin and CapG by pp60^{c-src}. A: Phosphorylation of fragmin. Fragmin (2.5 μM) was preincubated with PIP_2 (75 μM) and phosphorylated with pp60^{c-src} for 10 min. c: control: phosphorylation of fragmin without PIP_2. B: Phosphorylation of profilin. The phosphorylation of profilin (7 μM) was performed as described in A in the presence of a 30-fold molar excess of PIP_2. C: Phosphorylation of CapG. CapG (2.5 μM) was phosphorylated as described in A. The films were exposed for 8 h at room temperature.

made a comparative study of the stimulatory effects of different phospholipids. Of all phospholipids tested, PIP_2 displayed the highest activity (Fig. 3). In line with previous observations regarding the inhibition of gelsolin's F-actin severing activity by several phospholipids [41], PIP was able to mimic the effect of PIP_2 to a relatively high degree: indeed PIP caused a 16-fold increase in phospho-gelsolin formation at a molar excess of 30, whereas PIP_2 stimulated the activity by a factor of 25 under identical concentrations. PI, on the other hand, did not significantly increase gelsolin phosphorylation. PA showed only a weak stimulatory effect whereas PI, PS, PC, PE and ceramide did not enhance the phosphorylation of gelsolin at all (Fig. 3).

These findings underscore the specificity of PIP_2 and in particular the phosphate groups of the inositol moiety that seem to be required for a full-scale effect. Upon binding of the phospholipid, gelsolin probably undergoes a conformational change exposing the pp60^{c-src} target tyrosine residue(s).

3.5. Other actin-binding and PIP_2-binding proteins are similar substrates for pp60^{c-src}

To examine the specificity of pp60^{c-src} with regard to members of this family, we also investigated the ability of pp60^{c-src} to phosphorylate profilin, fragmin and CapG (three other actin- and PIP_2-binding proteins) and studied the effect of PIP_2 on phosphorylation. Under conditions similar to those described for gelsolin, we found that fragmin as well as profilin and CapG were phosphorylated by pp60^{c-src} (Fig. 4). In addition PIP_2, used at a 30-fold molar excess, significantly stimulated this activity. The stimulatory activity of PIP_2 towards these proteins was lower as compared to gelsolin. On average, we measured a 8- to 11-fold increase in phosphate incorporation (Fig. 4A,B,C). In the case of profilin and CapG the formation of two phosphotyrosine containing variants were observed. This was also verified by Western blot analysis with anti-phosphotyrosine antibodies (data not shown).

3.6. Gelsolin phosphorylation by PKC is stimulated by PIP_2

Because profilin phosphorylation by PKC [22] was stimulated by PIP_2, we investigated whether gelsolin could also be phosphorylated by PKC. Indeed, we found that gelsolin was phosphorylated and the phosphorylation was stimulated 10-fold in the presence of a 30-fold molar excess of PIP_2 (Fig. 5A). Also the autophosphorylation of PKC was stimulated by PIP_2. The phospho-amino acid pattern revealed phosphoserine as the major phospho-amino acid and to a lesser extent phosphothreonine (Fig. 5B).

4. Discussion

To our knowledge this is the first report demonstrating that gelsolin is a target for pp60^{c-src}. PIP_2 is necessary to obtain a significant degree of phosphorylation which amounts to 0.18 mol phosphate/mol gelsolin after 90 min in the presence of equimolar amounts of PIP_2 micelles. The strict dependence for PIP_2 most likely explains why phosphorylation of gelsolin previously escaped detection. Our data provide the biochemical background for earlier reports alluding to an association between pp60^{c-src} and gelsolin in osteoclasts [25]. The mechanism of PIP_2 stimulation is probably due to a conformational change in gelsolin induced by the phospholipid, resulting in the exposure of key tyrosine residues. The interaction of gelsolin with phospholipids has been reported to be specific for PIP_2 and PIP [40,41]. Our comparative study with anionic and neutral phospholipids reflects the same specificity.

Two-dimensional gel electrophoresis of phosphorylated gelsolin revealed two major and at least two minor phospho-variants, indicating that more than one tyrosine is involved (De Corte et al., unpublished results). PIP_2 does not act on the tyrosine kinase since the phosphorylation rate of RCM-lysozyme by pp60^{c-src} was not affected by the phospholipid. The specificity of pp60^{c-src} is nevertheless evidenced by the lack of tyrosine phosphorylation activity on gelsolin by pp56^{lck}, rendering it as unlikely that gelsolin is a substrate for pp56^{lck} in T lymphocytes, where both substrate and kinase are present [50,51]. This observation of substrate specificity runs parallel to the statement made by Cheng et al. [52] who argued that different tyrosine kinases assayed on different cdc2 peptide substrates displayed strict in vitro substrate specificities.

The amounts of PIP_2 that were used reflect actual physio-

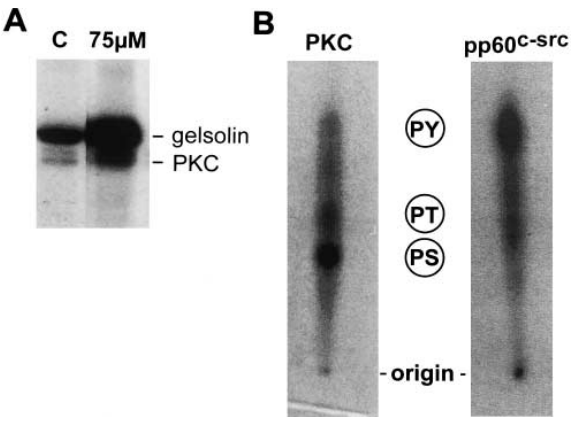

Fig. 5. Phosphorylation of gelsolin by PKC. A: Gelsolin (2.5 μM) was preincubated with the indicated PIP_2 concentration and phosphorylated with PKC for 10 min. c: control, gelsolin phosphorylated with PKC but without PIP_2. The autoradiogram was exposed for 8 h at room temperature. B: Phospho-amino acid analysis of gelsolin phosphorylated with PKC in the presence of PIP_2. pp60^{c-src} phosphorylation of gelsolin in the presence of PIP_2 is included for comparison. The phospho-amino acids were identified by their comigration with standards that were detected by ninhydrin staining (PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine). The autoradiogram was exposed for 3 h at room temperature.

logical concentrations [19] and considering the calculated K_m value ($\sim$6 μM), it is reasonable to speculate that gelsolin may represent a true substrate for pp60^{c-src} in various cell types. It is, however, likely that the phosphorylation occurs transiently and therefore may be difficult to detect in vivo. Significantly, half maximal stimulation of gelsolin phosphorylation only requires 15- to 20-fold molar excess of PIP_2, whereas half maximal inhibition of actin severing activity of gelsolin requires equimolar amounts of PIP_2 micelles ($\sim$80-fold molar excess) [19].

Based on our observations we postulate that pp60^{c-src} could be an additional regulator of the actin cytoskeleton through tyrosine phosphorylation of gelsolin. Many reports in the literature have emphasized a translocation event of pp60^{c-src} to and from the cytoskeleton. For example, it has been demonstrated that pp60^{c-src} translocates to the Triton X-100 insoluble cytoskeleton during platelet aggregation following thrombin stimulation [53,54]. Addition of platelet activating factor (PAF), however, causes pp60^{c-src} to translocate to the plasma membrane [55]. PIP_2 is known to dissociate the actin–gelsolin complex [56] and mediates uncapping of gelsolin from the barbed ends of actin filaments after stimulation of platelets with the thrombin receptor-activating peptide [20] and allowing the filaments to grow at this end. PIP_2 could convert gelsolin in a conformation favourable for phosphorylation by pp60^{c-src} and therefore giving new properties to gelsolin which will be recognized by future studies involving the identification of the phosphorylated tyrosine residues and functional assays.

Fragmin from *P. polycephalum*, mammalian profilin and CapG are also phosphorylated by pp60^{c-src} and as for gelsolin this phosphorylation is stimulated by PIP_2. PIP_2 is known to regulate the actin-binding properties of these proteins [46,57,58]. These findings may point to a more general mechanism involving tyrosine phosphorylation of other actin-binding proteins, with PIP_2 as the common denomenator [59]. Based on the structural and functional similarties between members of the gelsolin family, we hypothesize that severin from *Dictyostelium discoideum* and villin are also candidates for pp60^{c-src} phosphorylation. A similar role for PIP_2 may also apply to other cytoskeleton regulatory proteins involved in signal transduction pathways leading to microfilament rearrangements, such as vinculin [60] and α-actinin [61].

As previously shown [22], profilin phosphorylation by PKC was stimulated in the presence of PIP_2. In addition we found that this mechanism also applies to gelsolin. Thus it appears that several actin-binding proteins, once they have bound PIP_2, become good substrates for different protein kinases. Further analysis of these phosphorylations will help us to clarify their functional role and significance during agonist induced actin reorganization.

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