

## Signal transduction in neutrophil activation

### Phosphatidylinositol 3-kinase is stimulated without tyrosine phosphorylation

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Treatment of human neutrophils with the peptide f-Met-Leu-Phe (FMLP) results in neutrophil activation concomitant with stimulation of phosphatidylinositol (PtdIns) 3-kinase activity as measured by production of PtdIns-3,4,5-P<sub>3</sub> in [<sup>32</sup>P]orthophosphate labeled cells. Antiphosphotyrosine immunoprecipitates were assayed for PtdIns 3-kinase activity; essentially no activity was present in lysates from either stimulated or unstimulated cells. The 85 kDa regulatory subunit of PtdIns 3-kinase, which normally serves as a substrate for tyrosine kinases, was not detected by SDS-PAGE or Western blot analysis in antiphosphotyrosine immunoprecipitates. In addition, no radioactive band corresponding to PtdIns 3-kinase was observed by SDS-PAGE following antiPtdIns 3-kinase immunoprecipitations. However, immunoprecipitates using polyclonal antibodies against PtdIns 3-kinase showed high PtdIns 3-kinase activity in neutrophil lysates and the 85 kDa subunit of PtdIns 3-kinase was detected in Western blots; no differences in activity were observed in FMLP-stimulated and unstimulated cells. These results suggest that, in contrast to polypeptide growth factor signal transduction systems, the activation of PtdIns 3-kinase by FMLP does not require tyrosine phosphorylation.

Neutrophil activation; Tyrosine phosphorylation; Phosphatidylinositol 3-kinase; Signal transduction

#### 1. INTRODUCTION

Chemotactic agents such as the peptide *N*-formyl-Met-Leu-Phe (FMLP) and leukotriene B<sub>4</sub> activate neutrophils by stimulating motility and causing changes in cytoskeletal structure and contractile apparatus [1,2]. Changes in actin polymerization induced by FMLP have been shown to be concomitant with the formation of the novel phospholipid, phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P<sub>3</sub>) [3]. This lipid is one of three products of the enzyme PtdIns 3-kinase, a heterodimer of 110 kDa and 85 kDa subunits that has been implicated in growth factor signal transduction by associating with a number of proteins containing intrinsic or associated tyrosine kinase activities, including the receptors for platelet-derived growth factor (PDGF) [4–6], insulin [7,8], and colony stimulating factor-1 [9,10], the products of oncogenes *v-src* [11], *v-yes* [11], and *v-abl* [12], as well as the polyomavirus middle T antigen/pp60c-*src* complex [13]. The 85 kDa subunit of PtdIns 3-kinase is phosphorylated by tyrosine kinases, including the aforementioned receptors, in response to growth

factor stimulation; it is thought that phosphorylation of PtdIns 3-kinase is critical for activation of the kinase and the subsequent mitogenesis seen in stimulated cells [14]. Since the FMLP receptor is not a member of the tyrosine kinase family of receptors and still activates PtdIns 3-kinase [15,16], it was of interest to determine if tyrosine phosphorylation of PtdIns 3-kinase was responsible for stimulation of its activity. Results presented below suggest that tyrosine phosphorylation of PtdIns 3-kinase does not occur following neutrophil stimulation by FMLP. PtdIns 3-kinase activity is not present in antiphosphotyrosine immunoprecipitates, nor are bands corresponding to the 85 kDa subunit of the kinase present in SDS-PAGE analysis of the antiphosphotyrosine and antiPtdIns 3-kinase immunoprecipitates. However, PtdIns 3-kinase activity is stimulated by FMLP in neutrophils since the kinase products are detected following stimulation of cells by FMLP and since the kinase activity is observed in anti-PtdIns 3-kinase immunoprecipitates. Therefore it is apparent that although PtdIns 3-kinase activity is stimulated in neutrophils following treatment with FMLP, tyrosine phosphorylation of PtdIns 3-kinase is not necessary for this activation.

#### 2. EXPERIMENTAL

##### 2.1. Materials

Antiphosphotyrosine monoclonal antibodies and anti-PtdIns 3-kinase polyclonal antibodies were obtained from Upstate Biologicals,

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*Abbreviations:* FMLP, *N*-formyl-Met-Leu-Phe; PtdIns, phosphatidylinositol; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; gPtdIns, glycerophosphatidylinositol.

Inc. Protein A-Sepharose, aprotinin, sodium orthovanadate, phosphatidylinositol, butylated hydroxytoluene (BHT), phenylmethylsulfonyl fluoride (PMSF), and diisopropyl fluorophosphate (DFP) were products of Sigma Chemical Company, Inc. [ $\gamma$ - $^{32}$ P]ATP (6000 Ci/mmol), [ $^{32}$ P]orthophosphoric acid (HCl-free, 8500-9120 Ci/mmol), PtdIns-4-phosphate (inositol 2- $^3$ H(N), 10 Ci/mmol), and PtdIns-4,5-bisphosphate (inositol 2- $^3$ H(N), 10 Ci/mmol) were products of New England Nuclear. Flow-Scint IV liquid scintillator was obtained from Radiomatic Instruments and Chemical Co., Inc. Prestained SDS-PAGE molecular weight markers (high and low ranges) were products of Bio-Rad Laboratories, Inc. Milli-Q water (Millipore Corp.) was used for all aqueous solutions. All other reagents were of the highest quality commercially available.

### 2.2. Neutrophil isolation

Neutrophils were isolated from freshly drawn, heparin-treated human blood (25 ml) as follows. Blood was carefully layered onto 15 ml Mono-Poly Resolving Medium (Flow Laboratories, Inc.), and the resulting suspension centrifuged at 850  $\times$  g (30 min, 20°C). Plasma and monocytes were removed by aspiration, and the neutrophils were carefully removed and retained. Neutrophils were twice washed in PBS (without  $\text{Ca}^{2+}$ ), resuspended in 30 ml PBS, and quantitated using a Cell-Dyn 1600 Counter.

### 2.3. Phospholipid labeling and extraction

Neutrophils were labeled with [ $^{32}$ P]orthophosphate as described previously [17]. Neutrophils were washed in 30 mM HEPES buffer, pH 7.4, containing 110 mM NaCl, 10 mM KCl, 1 mM  $\text{MgCl}_2$ , and 10 mM glucose (buffer A) containing 1.53 mM  $\text{CaCl}_2$ . Cells were resuspended to a concentration of  $5 \times 10^7$ /ml in calcium-free buffer A containing 2 mg/ml BSA. [ $^{32}$ P]-Orthophosphoric acid (0.5 mCi/ml) was added and cells incubated at 37°C for 90 min. Cells were then washed three times in calcium-free buffer A, once in buffer A containing 1.53 mM  $\text{Ca}^{2+}$ , and split into 1 ml samples containing  $2 \times 10^7$  cells. Cells were preincubated at 37°C for 5 min and then were either treated with 1  $\mu$ l DMSO (unstimulated control) or 1  $\mu$ l DMSO containing FMLP (10 nM final concentration) for 60 s at 37°C, after which time the cell suspension was transferred to a solution of 3 ml  $\text{CHCl}_3/\text{MeOH}$  (1:2, v/v) containing 1 mg/ml BHT and 10  $\mu$ g/ml PtdIns/PtdIns-4-P/PtdIns-4,5-P<sub>2</sub> (1:1:1).  $\text{CHCl}_3$  and 2.4 N HCl (2.1 ml each) were added to the solution, which was then centrifuged. The lower  $\text{CHCl}_3$  phase was removed while the upper phase was washed 3 times with 1 ml  $\text{CHCl}_3$ . The chloroform washings were combined and washed with 0.5 ml  $\text{MeOH}/1$  N HCl (1:1, v/v). Following centrifugation, the lower  $\text{CHCl}_3$  phase was removed, taken to dryness on a Buchler Vortex-Evaporator, and resuspended in 100  $\mu$ l  $\text{CHCl}_3$  for thin-layer chromatography on 20  $\times$  20 cm Silica gel-60 plates (EM Science) impregnated with 1.2% potassium oxalate [17]. Plates were developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v). Radiolabeled phospholipids were detected by autoradiography. Migration of the products was compared to [ $^{32}$ P]PtdIns-3-P, [ $^{32}$ P]PtdIns-3,4-P<sub>2</sub>, and [ $^{32}$ P]PtdIns-3,4,5-P<sub>2</sub> formed by incubating [ $\gamma$ - $^{32}$ P]ATP and PtdIns/PtdIns-4-P/PtdIns-4,5-P<sub>2</sub>/phosphatidylserine (1:1:1:2, 0.2 mg/ml) with purified bovine brain PtdIns 3-kinase; standard PtdIns, PtdIns-4-P, and PtdIns-4,5-P<sub>2</sub> were co-chromatographed and visualized by  $I_2$  vapor.

Products of the PtdIns kinase reaction were confirmed by HPLC [18]. Phospholipids were deacylated in methylamine reagent and separated using a Whatman Partisphere SAX anion-exchange column as previously described [19]. A Radiomatic Model A-100 Flo-One/Beta on-line radioactivity detector was used to monitor the deacylated [ $^{32}$ P]enzyme products; [ $^3\text{H}$ ]gPtdIns-4-P and [ $^3\text{H}$ ]gPtdIns-4,5-P<sub>2</sub> were added as internal standards.

### 2.4. Phosphoprotein labeling and immunoprecipitation

Isolated human neutrophils were labeled with [ $^{32}$ P]orthophosphate (1 mCi/ $5 \times 10^7$  cells) as described above except that 1.0 mM sodium

orthovanadate was included in all buffers. Aliquots (0.5 ml) of the neutrophil suspension were then treated with either 1  $\mu$ l DMSO (unstimulated control) or 1  $\mu$ l DMSO containing FMLP (10 nM final concentration) for 60 s. Stimulation was quenched by addition of 1 ml inhibitor buffer (buffer A containing 1.53 mM  $\text{CaCl}_2$ , 100 mM NaF, 10 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM  $(\text{NH}_4)_2\text{MoO}_4$ , 1 mM iodoacetic acid, 1 mM benzamidine, and 1 mM sodium orthovanadate) [20]. Cells were then pelleted in a microcentrifuge, resuspended in ice-cold inhibitor buffer containing 0.5 mM DFP [21], 20  $\mu$ M leupeptin, and 20  $\mu$ M pepstatin, and incubated for 10 min on ice. Cells were pelleted by microcentrifugation, washed with 1 ml lysis wash buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , and 1 mM sodium orthovanadate) and were then lysed in 1 ml of 1% NP-40 lysis buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10% glycerol, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 1 mM PMSF, 1 mM sodium orthovanadate, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin at 4°C for 20 min. Insoluble material was removed by centrifugation at 10,000  $\times$  g for 10 min at 4°C. Antiphosphotyrosine antibody (5  $\mu$ g) or anti-PtdIns 3-kinase antisera (5  $\mu$ l) were incubated with the lysate supernatants for 2 h at 4°C. Immobilized protein A-Sepharose beads were used to precipitate the antibody-antigen complexes. Immunoprecipitates were washed twice with 1 ml of 0.1 M Tris, 0.5 M LiCl (pH 7.5). Following the second wash, 500  $\mu$ l of the resuspended immunoprecipitate was assayed for PtdIns 3-kinase activity as described below. Samples were microcentrifuged, and the resulting immunopellet from the remaining sample was boiled for 5 min in 100  $\mu$ l 4 $\times$  SDS-PAGE sample buffer (0.2 M Tris-HCl, pH 6.8, containing 40% glycerol, 4% SDS, 4%  $\beta$ -mercaptoethanol, and 0.025% Bromophenyl blue). To cell lysates not subjected to immunoprecipitation were added 250  $\mu$ l 4 $\times$  SDS-PAGE sample buffer; samples were boiled as above. The [ $^{32}$ P]proteins were separated by SDS-PAGE on a 7.5% gel. Gels were subjected to Western blot analysis as described below or dried in vacuo onto Whatman no. 3 paper and analyzed by autoradiography.

### 2.5. Western blot

The anti-PtdIns 3-kinase immunopellets were washed twice with 0.2 M Tris-HCl buffer, pH 7.5, containing 5 mM  $\text{MnCl}_2$ . The immunopellets were then boiled for 5 min with 30  $\mu$ l 4 $\times$  SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE (7.5% acrylamide); PtdIns 3-kinase isolated from bovine brain was used as a positive control. Proteins were transferred onto Hybond-ECL nitrocellulose (Amersham Corp.), and the membrane was then treated with blocking buffer (5% non-fat dry milk in 20 mM Tris-HCl, pH 7.5, containing 500 mM NaCl and 0.05% Tween 20). The membrane was incubated overnight with the rabbit anti-PtdIns 3-kinase primary antibody (1:500 dilution in blocking buffer containing 1% non-fat dry milk), and the blot was developed using the Amersham enhanced chemiluminescence detection method with donkey anti-rabbit IgG coupled to peroxidase as the secondary antibody.

### 2.6. Phosphatidylinositol 3-kinase activity

The immunopellets were assayed for PtdIns 3-kinase activity as described previously [13,22]. The immunoprecipitates were washed with 20 mM HEPES, pH 7.4, containing 10 mM  $\text{MgCl}_2$  and 25  $\mu$ M ATP, and were then resuspended in 40  $\mu$ l of the above buffer containing 32  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP. Reaction was initiated upon addition of 10  $\mu$ l PtdIns sonicated in 20 mM HEPES buffer, pH 7.4 (0.2 mg/ml final concentration). The samples were incubated at room temperature for 10 min with frequent mixing, after which time the reaction was quenched by addition of 40  $\mu$ l 1 N HCl. Lipids were extracted with addition of 80  $\mu$ l chloroform/methanol (1:1, v/v). The samples were centrifuged and the lower organic phase was applied to a silica gel TLC plate, which was developed in  $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}/\text{NH}_4\text{OH}$  (45:35:8.5:1.5, v/v). Plates were dried, and the kinase reaction visualized by autoradiography.

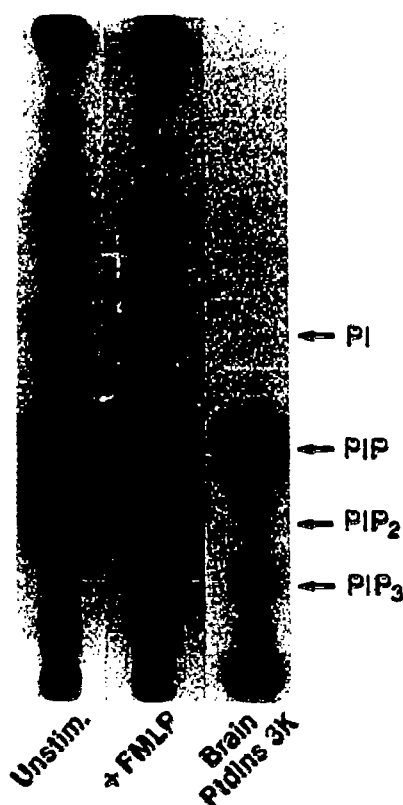


Fig. 1. Production of PtdIns-3,4,5- $P_3$  in FMLP-stimulated neutrophils. [ $^{32}P$ ]Orthophosphate labeled neutrophils were stimulated with 10 nM FMLP for 60 s. Phospholipids were extracted and separated by thin-layer chromatography as described in section 2. Migration of phosphatidylinositols was compared to PtdIns-3- $P$ , PtdIns-3,4- $P_2$ , and PtdIns-3,4,5- $P_3$  produced by purified bovine brain PtdIns 3-kinase; standards of PtdIns, PtdIns-4- $P$ , and PtdIns-4,5- $P_2$  were chromatographed and visualized by  $I_2$  vapor.

### 3. RESULTS

#### 3.1. Stimulation of PtdIns 3-kinase activity in activated neutrophils

Isolated human neutrophils were incubated with [ $^{32}P$ ]orthophosphate and then stimulated with FMLP (10 nM). Labeled phospholipids were extracted and analyzed by thin-layer chromatography (Fig. 1). Migration of the products was compared to [ $^{32}P$ ]PtdIns-3- $P$ , [ $^{32}P$ ]PtdIns-3,4- $P_2$ , and [ $^{32}P$ ]PtdIns-3,4,5- $P_3$  produced using purified bovine brain PtdIns 3-kinase; standard PtdIns, PtdIns-4- $P$ , and PtdIns-4,5- $P_2$  were co-chromatographed and visualized by  $I_2$  vapor. Stimulation of neutrophils with FMLP results in the rapid production of a novel [ $^{32}P$ ]phospholipid migrating just below PtdIns- $P_2$  that is absent in the unstimulated neutrophils. This compound co-migrated with PtdIns-3,4,5- $P_3$  produced by bovine brain PtdIns 3-kinase. Previous investigators have identified this compound as PtdIns-3,4,5- $P_3$  [15,17], and HPLC analysis following chemical deacyla-

tion of the phosphatidylinositols confirmed the identity of the novel phospholipid (Fig. 2). PtdIns-3,4,5- $P_3$  is not found in resting neutrophils (Fig. 2A) but is formed within one minute following stimulation by FMLP as evidenced by the peak eluting at 66 min (Fig. 2B) co-migrating with enzymatically synthesized PtdIns-3,4,5- $P_3$  (Fig. 2C). PtdIns-3,4- $P_2$  was also produced in neutrophils following FMLP stimulation. Production of PtdIns-3,4,5- $P_3$  and PtdIns-3,4- $P_2$  suggests the involvement of PtdIns 3-kinase in neutrophil activation; therefore, experiments were performed to investigate the role of this kinase.

#### 3.2. Protein phosphorylation

Antibodies to phosphotyrosine and PtdIns 3-kinase were used in immunoprecipitation experiments to measure differences in protein phosphorylation between cells stimulated with FMLP and unstimulated cells. Immunoprecipitates from both FMLP-treated and unstimulated cells were assayed for the presence of PtdIns 3-kinase activity as well as for differences in phosphoprotein content among the two cell types. This was accomplished by prelabeling cells with [ $^{32}P$ ]orthophosphate prior to immunoprecipitation in the presence of 1.0 mM sodium orthovanadate to inhibit tyrosine phosphatases. Fig. 3 depicts an autoradiograph comparing *in vitro* autophosphorylation with [ $^{32}P$ ]orthophosphate prelabeling of proteins isolated by antiphosphotyrosine immunoprecipitation. Cell lysates of unstimulated (lane 1) and FMLP-treated (lane 2) neutrophils show several phosphoprotein bands ranging from 50–200 kDa; there appears to be no difference in phosphoprotein content between the unstimulated and FMLP-treated neutrophils. Antiphosphotyrosine immunoprecipitates of unstimulated (lane 3) and FMLP-treated (lane 4) neutrophil lysates also show several prominent bands in the 50–180 kDa range; however, no [ $^{32}P$ ]phosphoprotein was observed at 85 kDa, corresponding to the regulatory subunit of PtdIns 3-kinase. A [ $^{32}P$ ]phosphoprotein corresponding to PtdIns 3-kinase was also absent in antiPtdIns 3-kinase immunoprecipitates of unstimulated (lane 5) and FMLP-treated (lane 6) neutrophil lysates. These findings suggest that PtdIns 3-kinase is not phosphorylated in response to neutrophil stimulation by FMLP.

#### 3.3. Phosphatidylinositol 3-kinase activity

Antiphosphotyrosine immunoprecipitates were assayed for the presence of PtdIns 3-kinase activity. As indicated in Fig. 4a, no enzymatic activity was present in both unstimulated and FMLP-treated cells, suggesting that tyrosine phosphorylation of PtdIns 3-kinase did not occur in formyl peptide-treated neutrophils. In contrast to antiphosphotyrosine immunoprecipitates, neutrophil lysate immunoprecipitates using polyclonal antisera against the 85 kDa subunit of PtdIns 3-kinase show very high PtdIns kinase activity in both stimulated

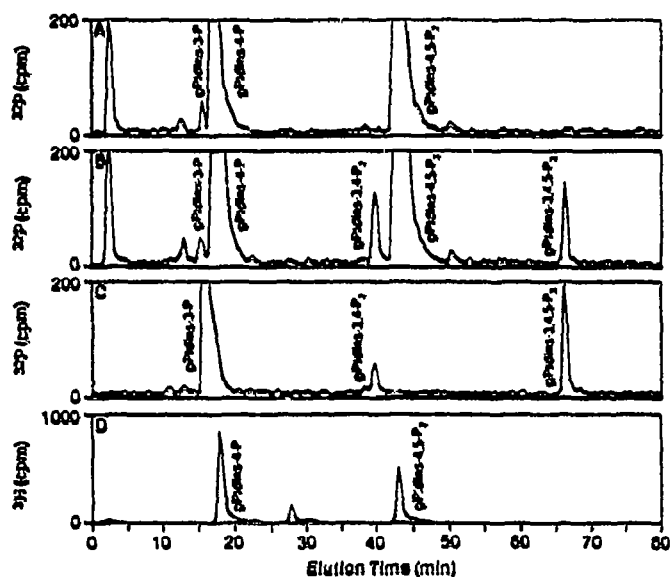


Fig. 2. HPLC analysis of deacylated PtdIns-phosphates. PtdIns-phosphates were extracted from TLC plates, chemically deacylated with methylamine, and subjected to anion exchange HPLC analysis using an on-line radiochemical detector as described in section 2. A, unstimulated neutrophils; B, FMLP-treated neutrophils; C, products of purified bovine brain PtdIns 3-kinase; D, [ $^3\text{H}$ ]PtdIns-4-P and [ $^3\text{H}$ ]PtdIns-4,5-P<sub>2</sub> internal standards.

and control cells (Fig. 4b); HPLC analysis of the deacylated lipid products of the PtdIns kinase assay confirms that the PtdIns kinase activity in these cells is due to the PtdIns 3-kinase rather than due to PtdIns 4-kinase (Fig. 4c). This indicates that neutrophils contain very significant PtdIns 3-kinase activity, but that tyrosine phosphorylation of PtdIns 3-kinase does not occur as indicated by the absence of the kinase in antiphosphotyrosine immunoprecipitates (Fig. 4a).

### 3.4. Anti-PtdIns 3-kinase Western blots

Western blot analysis using the polyclonal antisera against the SH2 domain of the 85 kDa regulatory subunit of PtdIns 3-kinase was performed on the immunoprecipitates obtained from using both the antiphosphotyrosine and anti-PtdIns 3-kinase antibodies (Fig. 5). The PtdIns 3-kinase is not found in antiphosphotyrosine immunoprecipitates, as indicated by the absence of the 85 kDa band in both the stimulated and unstimulated lysates (lanes 1 and 2). However, the 85 kDa subunit is present in the anti-PtdIns 3-kinase immunoprecipitate samples; no apparent differences are seen between stimulated and control samples (lanes 3 and 4). Several Western-positive bands are seen, especially around 95 kDa and 50 kDa, which represent components of the rabbit antibody (lane 5), but only the 85 kDa band in the anti-PtdIns 3-kinase immunoprecipitates correlates to PtdIns 3-kinase isolated from bovine

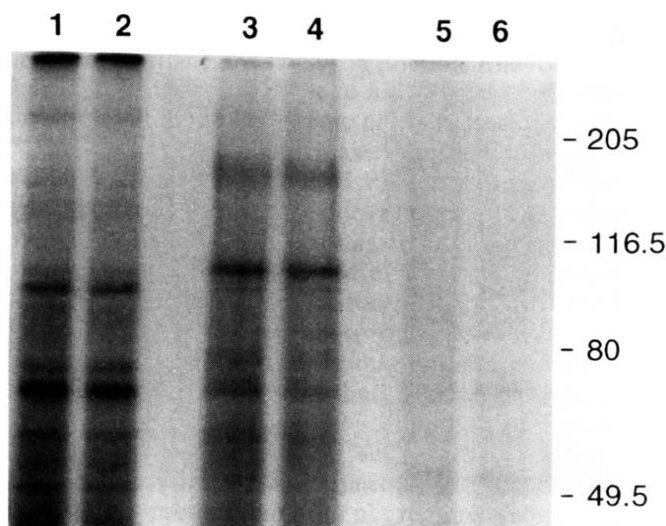


Fig. 3. [ $^{32}\text{P}$ ]Phosphoprotein analysis from neutrophil lysates. Neutrophils were labeled with [ $^{32}\text{P}$ ]orthophosphate as described in section 2. Lanes 1 and 2, cell lysate of unstimulated and FMLP-treated neutrophils, respectively; lanes 3 and 4, antiphosphotyrosine immunoprecipitates of cell lysates from unstimulated and FMLP-treated neutrophil lysates, respectively; lanes 5 and 6, anti-PtdIns 3-kinase immunoprecipitates of cell lysates from unstimulated and FMLP-treated neutrophil lysates, respectively. SDS-PAGE was performed on 7.5% acrylamide gels as described in section 2. Molecular weight markers: 205 kDa, myosin; 116.5 kDa,  $\beta$ -galactosidase; 80 kDa, bovine serum albumin; 49.5 kDa, ovalbumin.

brain, which was used as a positive control for the 85 kDa subunit (lane 6). The anti-PtdIns 3-kinase antisera was raised against the SH2 domain of the 85 kDa regulatory subunit; therefore, the 110 kDa subunit is not present in the Western blots since it is not recognized by the anti-PtdIns 3-kinase antisera. A faint band migrating to around 40 kDa in the anti-PtdIns 3-kinase immunoprecipitates is not present in the bovine brain PtdIns 3-kinase control lane, nor is it found in the antiphosphotyrosine immunoprecipitates. The identity of this protein is not known; it may represent another SH2-containing protein that was recognized by the polyclonal antisera, or may be due to cellular proteolytic processing of the 85 kDa subunit of the kinase. However, the absence of PtdIns 3-kinase in the antiphosphotyrosine immunoprecipitates further substantiates that although the PtdIns 3-kinase is present in the neutrophil, tyrosine phosphorylation of the kinase does not occur.

## 4. DISCUSSION

PtdIns 3-kinase is an enzyme that associates with a number of proteins that have intrinsic or associated tyrosine kinase activities including the receptors for PDGF [4,5,6], insulin [7,8], and colony-stimulating factor-1 [9,10], the products of oncogenes *v-src* [11], *v-yes*

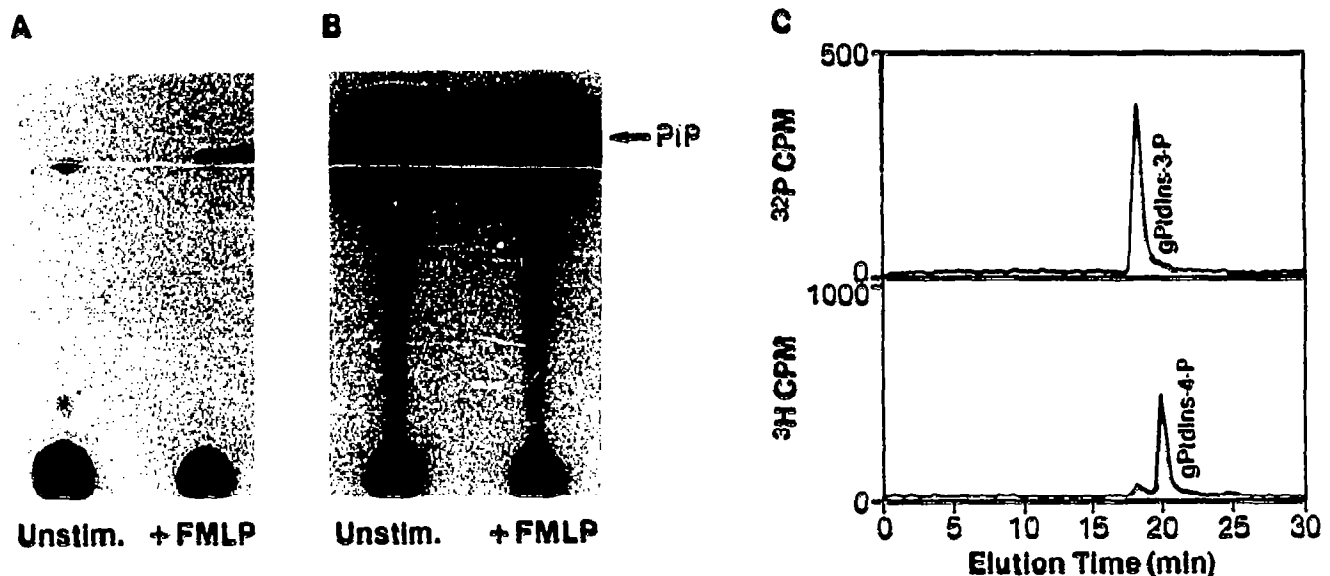


Fig. 4. Autoradiograph of PtdIns 3-kinase assay. A, antiphosphotyrosine immunoprecipitates; B, anti-PtdIns 3-kinase immunoprecipitates. Immunoprecipitates were assayed with PtdIns and [ $^{32}$ P]ATP as described in section 2. C, identity of the radioactive enzymatic product as PtdIns-3-P was confirmed by HPLC analysis following chemical deacylation (upper panel) by comparison to an internal standard of [ $^3$ H]gPtdIns-4-P (lower panel).

[11], and *v-abl* [12], as well as the polyomavirus middle T antigen/pp60c-src complex [13]. The enzyme is a heterodimer of 110 kDa and 85 kDa subunits [23]; the 85 kDa subunit binds to tyrosine kinases and serves as one of its substrates [24–26]. Mutants of the PDGF-receptor that lack the PtdIns 3-kinase binding site fail to have associated PtdIns 3-kinase activity, and cells expressing these mutant receptors fail to show increased DNA synthesis and cell division, suggesting an important role for PtdIns 3-kinase in mitogenesis [27,28]. To date, however, the physiological role of PtdIns 3-kinase or its products has not been elucidated, although there is some suggestion about the role of the PtdIns-3-phosphates involved in the regulation of cytoskeletal structure [3,29].

Tyrosine phosphorylation of PtdIns 3-kinase is thought to be essential for its activation in polypeptide growth factor-receptor systems [14], perhaps involving translocation of the PtdIns 3-kinase to the cell membrane [30]. Although the FMLP receptor does not have intrinsic tyrosine kinase activity, it is possible that a coupled cytosolic tyrosine kinase could be involved in PtdIns 3-kinase activation. It has previously been demonstrated that tyrosine phosphorylation occurs in activated human neutrophils [31–33], but none of these studies have linked tyrosine phosphorylation with PtdIns 3-kinase activation. Experiments were therefore performed to examine whether PtdIns 3-kinase is phosphorylated on tyrosine following stimulation with FMLP.

In contrast to experiments performed in fibroblasts or smooth muscle cells [18,19], antibodies raised against phosphotyrosine do not immunoprecipitate PtdIns 3-

kinase activity in neutrophils, suggesting that the enzyme is not phosphorylated on tyrosine in activated neutrophils. PtdIns 3-kinase activity was absent in antiphosphotyrosine immunoprecipitates from both unstimulated and FMLP-treated neutrophils (Fig. 4a), and the 85 kDa protein representing the regulatory subunit of PtdIns 3-kinase was not present in cell lysates or antiphosphotyrosine immunoprecipitates using SDS-PAGE analysis of the  $^{32}$ P-labeled proteins (Fig. 3), or anti-PtdIns 3-kinase Western blots from antiphosphotyrosine immunoprecipitations (Fig. 5). However, it is evident that PtdIns 3-kinase is present in the neutrophil, as demonstrated by the production of PtdIns-3,4,5-P<sub>3</sub> upon stimulation with FMLP (Figs. 1 and 2), the ability to measure PtdIns 3-kinase activity in anti-PtdIns 3-kinase immunoprecipitates (Fig. 4), and the presence of the 85 kDa subunit in Western blots following anti-PtdIns 3-kinase immunoprecipitations (Fig. 5). While it is certainly possible that tyrosine phosphorylation occurs on a small fraction of PtdIns 3-kinase in neutrophils, similar experiments performed in 3T3 fibroblasts support the findings presented in this report. Stimulation of 3T3 cells with PDGF results in the presence of PtdIns 3-kinase in antiphosphotyrosine immunoprecipitations; this activity is absent in unstimulated cells. The amount of PtdIns 3-kinase activity in antiphosphotyrosine immunoprecipitations appears to constitute less than 1% of the total PtdIns 3-kinase activity when compared to anti-PtdIns 3-kinase immunoprecipitations from 3T3 cell lysates. Thus, although only a small fraction of PtdIns 3-kinase activity in 3T3 cells is present in antiphosphotyrosine immunoprecipitates, it is clearly and significantly present compared to unstimulated

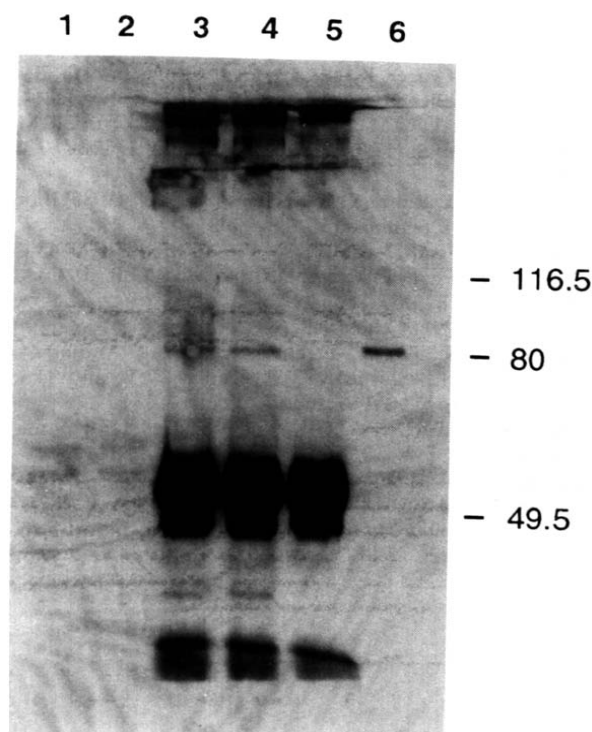


Fig. 5. Western blot of PtdIns 3-kinase in immunoprecipitates. Lanes 1 and 2, unstimulated and FMLP-treated lysate from antiphosphotyrosine immunoprecipitates; lanes 3 and 4, unstimulated and FMLP-treated lysate from anti-PtdIns 3-kinase immunoprecipitates; lane 5, rabbit anti-PtdIns 3-kinase antibody; lane 6, PtdIns 3-kinase isolated from bovine brain.

control. This is in contrast to the findings presented in this report for the neutrophil. Antiphosphotyrosine immunoprecipitates show no stimulation of PtdIns 3-kinase activity in FMLP stimulated neutrophils. Essentially no PtdIns 3-kinase activity is present in both unstimulated and FMLP-treated neutrophils. These data, as well as the absence of PtdIns 3-kinase in Western blots of antiphosphotyrosine immunoprecipitates and the absence of the kinase in antiphosphotyrosine immunoprecipitates from  $^{32}\text{P}$ -labeled cell lysates, strongly suggest that tyrosine phosphorylation of PtdIns 3-kinase does not occur during neutrophil activation.

On the surface, it would appear that the presence of PtdIns 3-kinase in neutrophils and its activation following FMLP stimulation would be contrary to previous reports suggesting a direct link between PtdIns 3-kinase activity and mitogenesis [27,28] since neutrophils are nonproliferating cells and the chemotactic peptide receptor is not a tyrosine kinase. However, the production of PtdIns-3,4,5- $\text{P}_3$  is concomitant with changes in actin polymerization in neutrophils stimulated with FMLP [3], and changes in actin cytoskeletal structure also occur during mitogenesis [34-36]. Furthermore, these cytoskeletal changes are mediated in part by PtdIns-4,5- $\text{P}_2$ , which has been demonstrated to bind to

prolactin, gelsolin, and villin and therefore promote actin polymerization [37-39]. A possible role for PtdIns-3,4,5- $\text{P}_3$  production may be to induce actin polymerization in response to mitogenic or chemotactic stimuli.

In summary, PtdIns 3-kinase activity is stimulated in neutrophils treated with FMLP, but tyrosine phosphorylation of PtdIns 3-kinase does not occur. The presence of PtdIns 3-kinase activity has been demonstrated by measuring activity in anti-PtdIns 3-kinase immunoprecipitates as well as the isolation of its product PtdIns-3,4,5- $\text{P}_3$  from stimulated neutrophils. However, the activity is not present in antiphosphotyrosine immunoprecipitates, again supporting that tyrosine phosphorylation is not necessary for the stimulation of PtdIns 3-kinase activity. These results suggest that in nonmitogenic cells, an alternate mechanism for PtdIns 3-kinase activation is present that does not require tyrosine phosphorylation but still enables responses such as actin polymerization to occur.

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