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Signal transduction in neutrophil activation

Phosphatidylinositol 3-kinase is stimulated without tyrosine phosphorylation

Chris J. Vlahos and William F. Matter

Cardiovascular Research, Lilly Research Laboratories, Indianapolis IN 46285-0403, USA

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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_3 in [^{32}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 85kDa 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 B4 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_3) [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 phosporylation of PtdIns 3-kinase is not necessary for this activation.

Correspondence address: C. Vlahos, Cardiovascular Research, Lilly Research Laboratories, Indianapolis, IN 46285-0403, USA. Fax: (1) (317) 276 9722.

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.

2. EXPERIMENTAL

2.1. *Materials*

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

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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. [γ-^{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 × *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 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 $MgCl_2$, and 10 mM glucose (buffer A) containing 1.53 mM $CaCl_2$. Cells were resuspended to a concentration of 5×10^7/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 Ca^{2+}, and split into 1 ml samples containing 2×10^7 cells. Cells were preincubated at 37°C for 5 min and then were either treated with 1 μl DMSO (unstimulated control) or 1 μl 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 $CHCl_3$/MeOH (1:2, v/v) containing 1 mg/ml BHT and 10 μg/ml PtdIns/PtdIns-4-P/PtdIns-4,5-P_2 (1:1:1). $CHCl_3$ and 2.4 N HCl (2.1 ml each) were added to the solution, which was then centrifuged. The lower $CHCl_3$ phase was removed while the upper phase was washed 3 times with 1 ml $CHCl_3$. The chloroform washings were combined and washed with 0.5 ml MeOH/1 N HCl (1:1, v/v). Following centrifugation, the lower $CHCl_3$ phase was removed, taken to dryness on a Buchler Vortex-Evaporator, and resuspended in 100 μl $CHCl_3$ for thin-layer chromatography on 20 × 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_2, and [^{32}P]PtdIns-3,4,5-P_3 formed by incubating [γ-^{32}P]ATP and PtdIns/PtdIns-4-P/ PtdIns-4,5-P_2/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_2 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}H]gPtdIns-4-P and [^{3}H]gPdIns-4,5-P_2 were added as internal standards.

2.4. *Phosphoprotein labeling and immunoprecipitation*

Isolated human neutrophils were labeled with [^{32}P]orthophosphate (1 mCi/5 × 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 μl DMSO (unstimulated control) or 1 μl 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 $CaCl_2$, 100 mM NaF, 10 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM $(NH_4)_2MoO_4$, 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 μM leupeptin, and 20 μM 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 $MgCl_2$, 1 mM $CaCl_2$, 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 $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 4°C for 20 min. Insoluble material was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. Antiphosphotyrosine antibody (5 μg) or anti-PtdIns 3-kinase antisera (5 μl) 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 μl 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 μl 4× SDS-PAGE sample buffer (0.2 M Tris-HCl, pH 6.8, containing 40% glycerol, 4% SDS, 4% β-mercaptoethanol, and 0.025% Bromophenyl blue). To cell lysates not subjected to immunoprecipitation were added 250 μl 4× 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 $MnCl_2$. The immunopellets were then boiled for 5 min with 30 μl 4× 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 $MgCl_2$ and 25 μM ATP, and were then resuspended in 40 μl of the above buffer containing 32 μCi [γ-^{32}P]ATP. Reaction was initiated upon addition of 10 μl 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 μl 1 N HCl. Lipids were extracted with addition of 80 μl 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 $CHCl_3$/MeOH/H_2O/NH_4OH (45:35:8.5:1.5, v/v). Plates were dried, and the kinase reaction visualized by autoradiography.

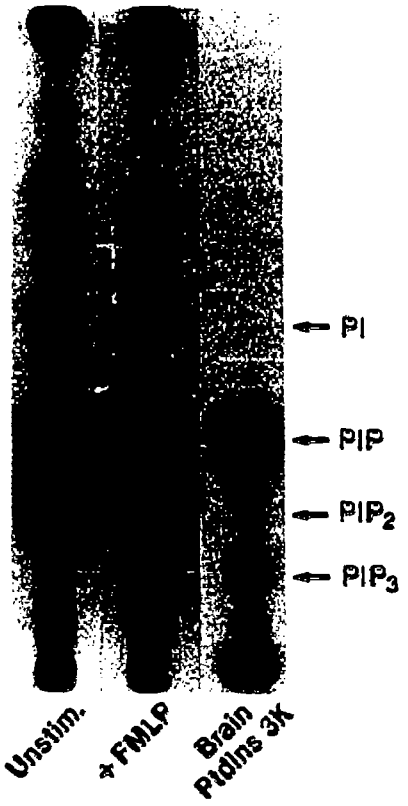

Fig. 1. Production of PtdIns-3,4,5-P_3 in FMLP-stimulated neutrophils. [^{32}P]Orthophosphate labeled neutrophils were stimulated with 10 nM FMLP for 60 s. Phospholipids were extracted and separated by thin-layer chromatography as described in section 2. Migration of phosphatidylinositols was compared 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 deacylation 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}]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

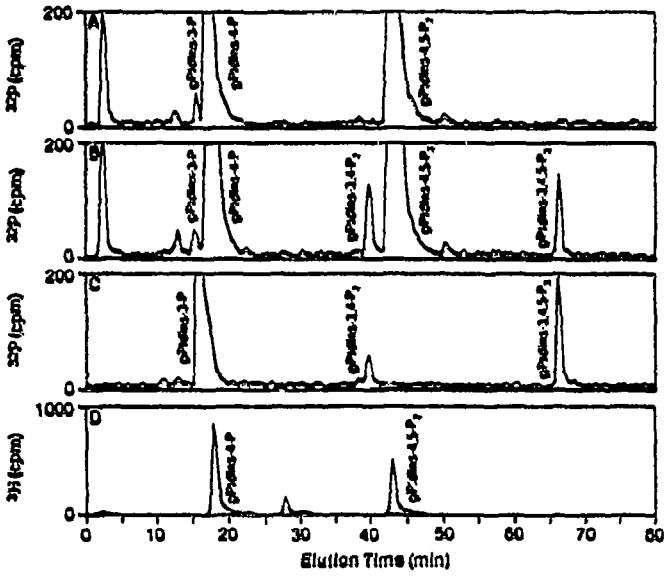

Fig. 2. HPLC analysis of deacylated PtdIns-phosphates. PtdIns-phosphates were extracted from TLC plates, chemically deacylated with methylamine, and subjected to anion exchange HPLC analysis using an on-line radiochemical detector as described in section 2. A, unstimulated neutrophils; B, FMLP-treated neutrophils; C, products of purified bovine brain PtdIns 3-kinase; D, [^{3}H]gPtdIns-4-P and [^{3}H]gPtdIns-4,5-P_2 internal standards.

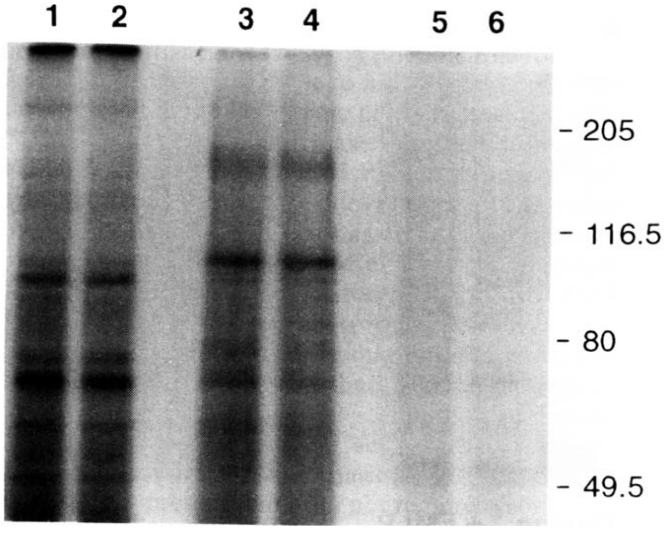

Fig. 3. [^{32}P]Phosphoprotein analysis from neutrophil lysates. Neutrophils were labeled with [^{32}P]orthophosphate as described in section 2. Lanes 1 and 2, cell lysate of unstimulated and FMLP-treated neutrophils, respectively; lanes 3 and 4, antiphosphotyrosine immunoprecipitates of cell lysates from unstimulated and FMLP-treated neutrophil lysates, respectively; lanes 5 and 6, antiPtdIns 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, β-galactosidase; 80 kDa, bovine serum albumin; 49.5 kDa, ovalbumin.

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 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*

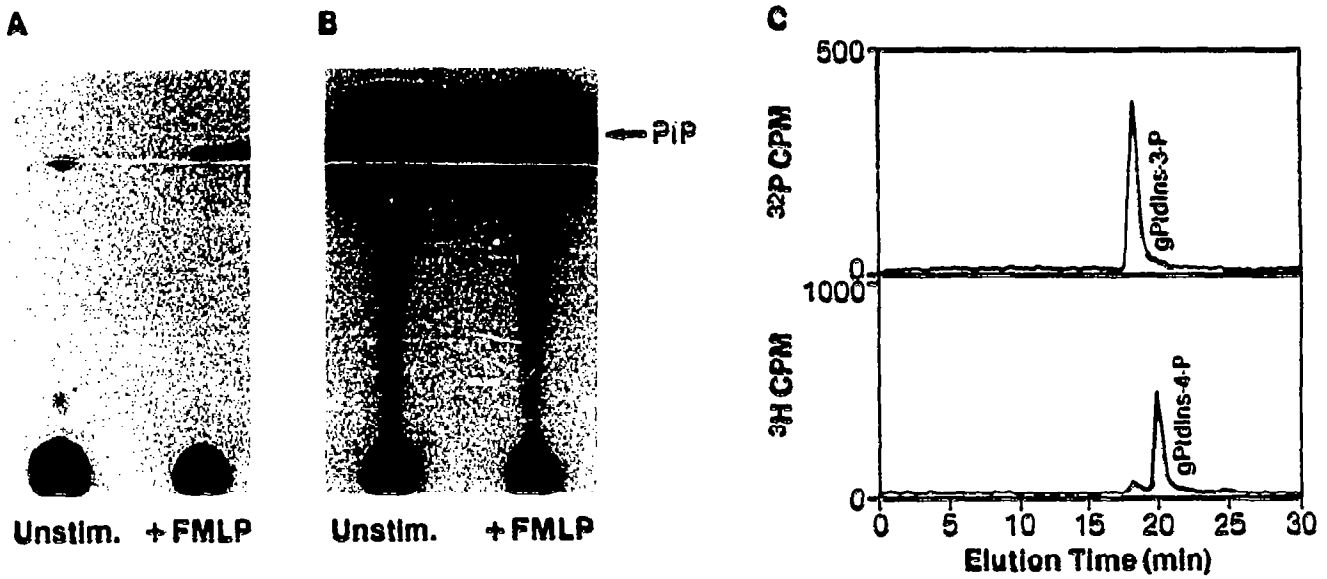

Fig. 4. Autoradiograph of PtdIns 3-kinase assay. A, antiphosphotyrosine immunoprecipitates; B, anti-PtdIns-3-kinase immunoprecipitates. Immunopellets were assayed with PtdIns and [^{32}P]ATP as described in section 2. C, identity of the radioactive enzymatic product as PtdIns-3-P was confirmed by HPLC 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_3 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

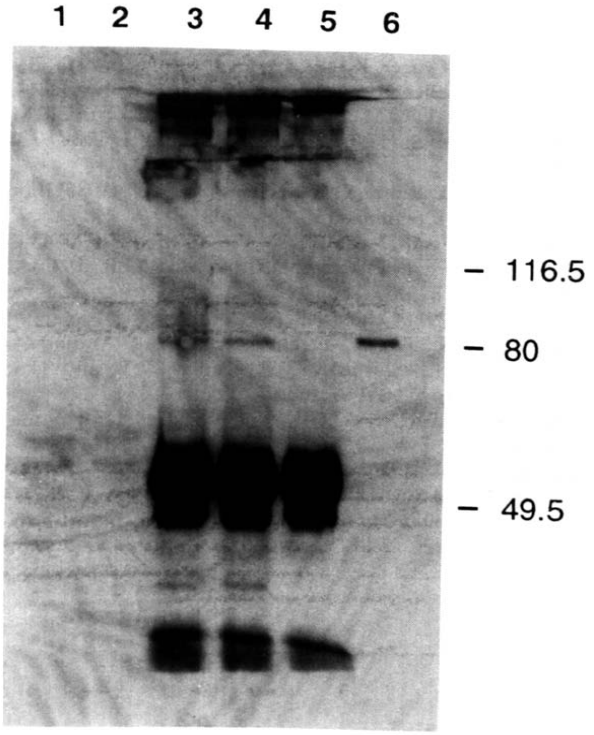

Fig. 5. Western blot of PtdIns 3-kinase in immunopellets. Lanes 1 and 2, unstimulated and FMLP-treated lysate from antiphosphotyrosine immunoprecipitates; lanes 3 and 4, unstimulated and FMLP-treated lysate from anti-PtdIns 3-kinase immunoprecipitates; lane 5, rabbit anti-PtdIns 3-kinase antibody; lane 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}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-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-P_2, which has been demonstrated to bind to profilactin, gelsolin, and villin and therefore promote actin polymerization [37–39]. A possible role for PtdIns-3,4,5-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-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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