Volume 329, number 3, 273–276 FEBS 12906 August 1993
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Lysophosphatidic acid induces tyrosine phosphorylation and activation of MAP-kinase and focal adhesion kinase in cultured Swiss 3T3 cells

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Received 6 July 1993

Lysophosphatidic acid (LPA) added to serum-starved Swiss 3T3 cells induced, in a time- and concentration-dependent manner, tyrosine phosphorylation of multiple proteins, including proteins of 43, 64, 88 kDa and a group of proteins between 110 and 130 kDa. Among them, two proteins, p43 and p120, were identified as mitogen-activated protein kinase (MAP-kinase) and focal adhesion kinase (FAK), respectively, by immunoprecipitation and immunoblot analysis. Tyrosine phosphorylation of p64 peaked at 1 min and declined rapidly, whereas that of MAP-kinase and FAK peaked at 5 and 10 min after the addition of LPA, respectively. The activity of MAP-kinase determined as phosphorylation of myelin basic protein increased transiently about 3-fold at 5 min, and correlated with tyrosine phosphorylation. These results indicate that tyrosine phosphorylation of these proteins is a part of the signal transduction by LPA and may be involved in its mitogenic responses.

Lysophosphatidic acid; Protein tyrosine phosphorylation; Protein tyrosine kinase; MAP-kinase, Focal adhesion kinase; Signal transduction

1. INTRODUCTION

Lysophosphatidic acid (LPA) evokes a variety of biological actions, such as platelet aggregation, smooth muscle contraction, induction of shape changes in neuronal cells, and cell proliferation in fibroblasts (see [1] for review). Although it is a simple phospholipid, LPA acts on a cell surface receptor to stimulate phosphoinositide metabolism and inhibit adenylate cyclase in a GTP-dependent manner [2,3]. The putative receptor for LPA has been identified as a membrane protein of 38–40 kDa by photoaffinity labeling with a photoreactive LPA analogue [4]. LPA is rapidly produced in activated platelets [5,6] and possibly secreted. Recently LPA was described as a normal constituent in serum and responsible for the majority of its growth-promoting activity [7,8]. When added to quiescent Swiss 3T3 cells, it characteristically induces formation of stress fiber and focal adhesion [8]. Recently it was reported that LPA stimulates protein tyrosine phosphorylation in fibroblasts [9]. However, details of the tyrosine-phosphorylated proteins remain to be characterized and the relevance of this tyrosine phosphorylation to growth promotion and cytoskeletal changes by LPA has not been clarified. To elucidate these issues, we examined and identified some of proteins tyrosine phosphorylated in LPA-stimulated quiescent Swiss 3T3 cells.

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Abbreviations: LPA, lysophosphatidic acid; MAP-kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; ITS, insulin-transferrin-sodium selenite; MBP, myelin basic protein; TBS, Tris-buffered saline.

2. MATERIALS AND METHODS

2.1. *Materials*

LPA (oleoyl-*sn*-glycero-3-phosphate) was obtained from Sigma Rabbit polyclonal anti-phosphotyrosine antibody and anti-p120 (FAK) monoclonal antibody were purchased from Zymed Laboratories and Upstate Biotechnology, respectively. [^{125}I]Protein A (86.7 mCi/ mg) and [^{32}P]ATP (3,000 Ci/mmol) were purchased from ICN Biomedicals and DuPont-New England Nuclear, respectively. Rabbit anti-mouse IgG was obtained from Seikagaku Co. (Tokyo). Insulin-transferrin-sodium selenite (ITS) supplement was obtained from Boehringer-Mannheim Biochimica. Protein A–Sepharose was purchased from Pharmacia. Anti-peptide antibodies against MAP-kinases, 1C for ERK1 and 2Y for ERK2, were produced and characterized as described [10].

2.2. *Cell culture and LPA stimulation*

Swiss 3T3 mouse fibroblasts were obtained from the Japanese Cancer Research Resource Bank and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (Whittaker Bioproducts) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) in a humidified atmosphere containing 5% CO_2. Cells were seeded at a density of 1.0×10^5 per well in a 6-well plate (Falcon) and cultured in the complete medium for 3–4 days. Confluent cells were then washed twice with Dulbecco's phosphate-buffered saline without magnesium chloride and calcium chloride (PBS(−)) pre-warmed at 37°C, and rendered quiescent by incubating in serum-free medium (a 1:1 mixture of DMEM and F12 Ham's medium containing 5 mg/ml bovine serum albumin (BSA) and 0.05% of the ITS supplement) for 12 h. Various concentrations of LPA were then added to the culture, and incubation was carried out at 37°C in

a CO_2 incubator for the indicated time. LPA was first dissolved in 20% ethyl alcohol at 10 mM concentration and diluted with serum-free medium. After incubation, the plate was immediately chilled on ice, the medium was aspirated and the cells were washed once with ice-cold PBS(−). Cells were then scraped with a rubber policeman and sonicated in 200 μl of modified RIPA buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EGTA, 1 mM PMSF, 100 U/ml aprotinin, 5 μM leupeptin and 10 μg/ml α_2-macroglobulin) for immunoblotting and immunoprecipitation, or in 400 μl of the same solution without the detergents for kinase assay.

2.3 *Immunoprecipitation and immunoblotting*

30 μl of the cell extracts was mixed with 6 μl of 5× Laemmli sample buffer (final concentration of mercaptoethanol, 5%) and boiled for 5 min. Samples were then subjected to SDS-PAGE in an 8 or 10% gel. Proteins were then transferred to Immobilon membranes (Millipore). Membranes were blocked with 5% BSA in Tris-buffered saline (TBS) and incubated with the anti-phosphotyrosine antibody or anti-MAP-kinase 2Y antibody in TBS–0.5% BSA overnight at room temperature. Membranes were then washed three times with 0.05% Tween-20 in TBS. Immunoreactive bands were visualized by incubation with [^{125}I]protein A diluted 10,000-fold in TBS–0.05% Tween 20, and analyzed by autoradiography or by a Biomage analyzer BAS2000 (FUJIX, Tokyo).

For immunoprecipitation of FAK, 70 μl of cell extracts was incubated with 2 μl of the anti-p120 antibody at 4°C overnight. Rabbit polyclonal anti-mouse IgG pre-coupled to protein A–Sepharose was then added. The mixture was incubated for 1 h at 4°C, and centrifuged at 1,000 × *g* for 3 min. For immunoprecipitation of MAP-kinase, 30 μl of cell lysate was denatured by heating at 80°C for 3 min in the presence of 0.5% SDS. The sample was diluted with 5 vols. of the modified RIPA buffer and incubated overnight with 2Y antiserum at a 1.50 dilution. Immunocomplexes were recovered by adsorption on protein A–Sepharose beads and centrifuged as described above. Immunoprecipitates were washed three times with 300 μl of the modified RIPA buffer, suspended in Laemmli buffer containing 5% mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE and immunoblotting as described above.

2.4. *Assays*

MAP-kinase activity was measured using myelin basic protein (MBP) as a substrate as described previously [10]. 2–5 μl of cell extracts was used in a total volume of 25 μl. Under this condition, phosphorylation of MBP was proportional to the sample volume. Kinase assay in a MBP-containing gel was performed according to the method of Kameshita and Fujisawa [11]. Protein contents were determined according to the method of Bradford with BSA as a standard [12].

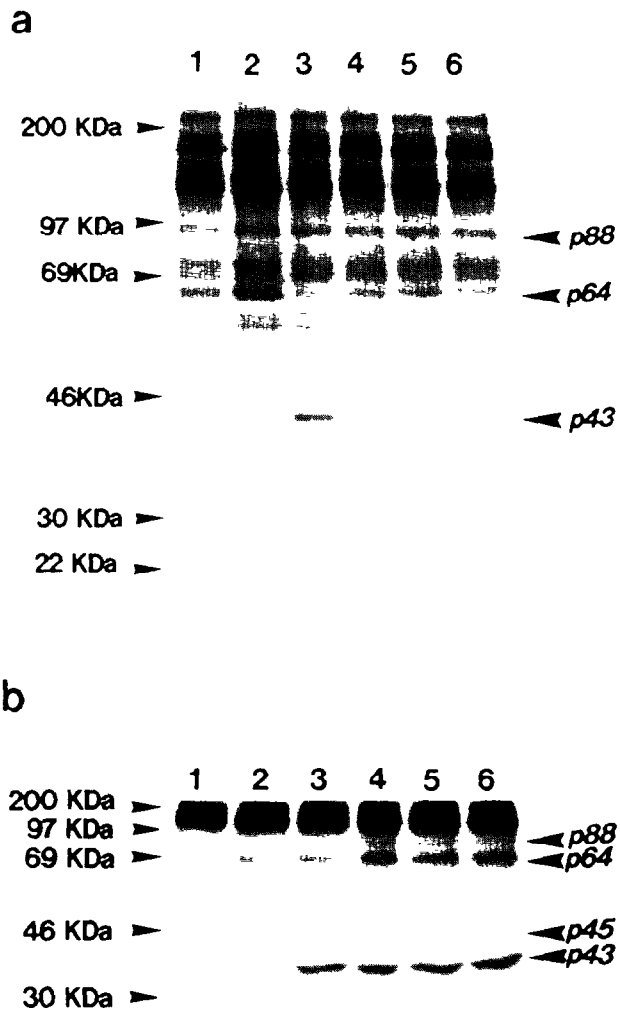


Fig. 1. Time–course (a) and concentration-dependency (b) of LPA-induced protein tyrosine phosphorylation in cultured Swiss 3T3 cells. Cells were incubated with 2 μM LPA for (a) various times ((min) lane 1, 0; 2, 1; 3, 5; 4, 10; 5, 30; 6, 60) or with (b) various concentrations ((nm) lane 1, 0; 2, 20; 3, 70; 4, 200; 5, 700; 6, 2,000) of LPA for 5 min. Cells were extracted and analyzed as described in section 2. Note that a doublet of bands, p43 and p45, was observed in panel b

3. RESULTS AND DISCUSSION

Fig. 1 shows the time–course and concentration-dependency of LPA-induced protein tyrosine phosphorylation. LPA stimulated tyrosine phosphorylation of multiple proteins in cultured Swiss 3T3 cells, including a broad band of M_r 110,000–130,000, and bands of M_r 88,000 (p88), 64,000 (p64) and 43,000 (p43). Labelling of these bands was specifically blocked by the addition of an excess amount of phosphotyrosine during the immunoreaction (data not shown), indicating that the labelling was indeed due to tyrosine phosphorylation. These bands showed a characteristic time–course of phosphorylation. Phosphorylation of p43, sometimes seen as a doublet of p43 and p45 (Fig. 1b), was detected 1 min after the addition of LPA, peaked at 5 min and declined quickly thereafter. On the other hand, the phosphorylation of p64 and p88, which were already present, albeit weakly, before the addition of LPA, increased quickly at 1 min and returned to basal levels. As shown in Fig. 1b, the phosphorylation occurred in a LPA concentration-dependent manner and reached a maximum at 200 nM. Tyrosine phosphorylation observed with LPA was similar to that induced by bombesin, vasopressin, endothelin or bradykinin in Swiss 3T3 cells [13,14], except that the phosphorylation of p43 and p64 was not reported for these peptides.

The identities of some of these tyrosine-phosphorylated proteins were examined by immunoprecipitation and immunoblotting analysis using

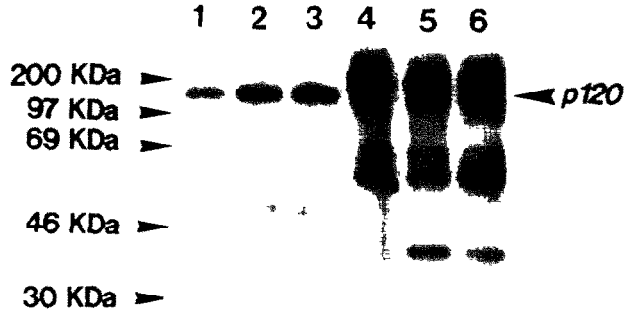


Fig. 2. Immunoprecipitation and identification of p120 FAK. Cells were incubated with 2 μM LPA for various times. Cell extracts were prepared and immunoprecipitation with the anti-p120 antibody was carried out as described in section 2. Immunoprecipitates were then analyzed by SDS-PAGE and by immunoblotting with the anti-phosphotyrosine antibody. Immunoprecipitates of cells before LPA addition (lane 1) and at 5 and 10 min after LPA addition (lanes 2 and 3, respectively) are shown. The corresponding supernatants were shown in lanes 4–6.

specific antibodies. Immunoprecipitation using the anti-p120 monoclonal antibody specific for p120 FAK revealed a single tyrosine-phosphorylated protein of 120 kDa (Fig. 2). Since its phosphorylation, already present at 0 min, was increased at 5 and 10 min after the LPA addition (Fig. 2), LPA induced tyrosine phosphorylation of FAK in these cells. Treatment of cell extracts with the anti-ERK2 antibody, 2Y, specifically precipitated the p43 tyrosine-phosphorylated band, as shown by the immunoblots in Fig. 3. Parallel blots of the immunoprecipitates with the anti-MAP-kinase antibody showed that the amount of the protein did not change during the incubation (data not shown). The identity of p43 was further verified by measuring the activity of

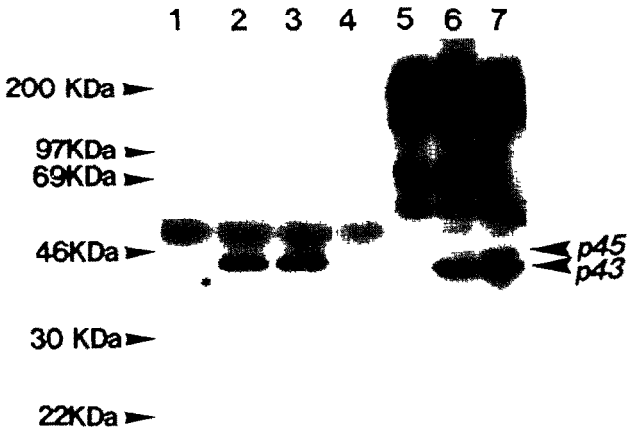
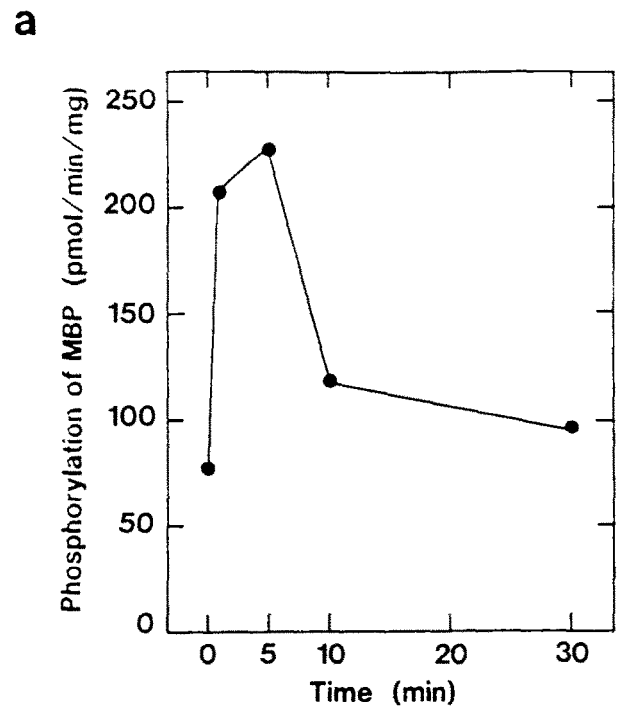


Fig. 3. Identification of p43 as MAP-kinase. Cells were incubated with 2 μM LPA for various times (lanes 1 and 5, 0 min; lanes 2 and 6, 1 min; lanes 3, 4 and 7, 5 min). Cell extracts were prepared and immunoprecipitation with anti-MAP-kinase (ERK2) antibody 2Y was carried out as described in section 2. Immunoprecipitates (lanes 1–3) and total cell lysates (lanes 5–7) were then analyzed by SDS-PAGE and by immunoblotting with the anti-phosphotyrosine antibody. Lane 4 shows immunoprecipitates with normal rabbit serum. Note that only p43 was precipitated



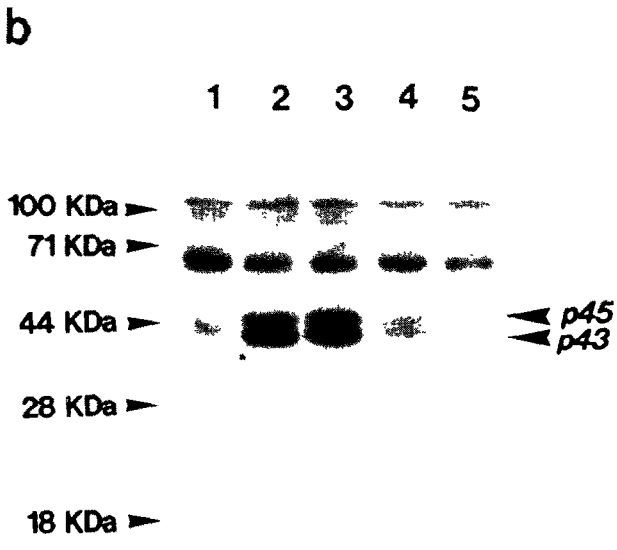


Fig. 4. LPA-induced MAP-kinase activation. Cells were incubated with 2 μM LPA for the indicated times. Cells were extracted and the MAP-kinase activity in the cell extracts was measured in (a) test tubes or (b) the MBP-containing gel (lane 1, 0 min; 2, 1 min; 3, 5 min; 4, 10 min; 5, 30 min) as described in section 2. Note that the enzyme activity was detected in both the p43 and p45 bands in the in-gel assay.

MAP-kinase in cell extracts. This activity, determined by MBP phosphorylation, increased transiently at 1–5 min after the LPA addition (Fig. 4a), and the in-gel phosphorylation analysis performed in parallel showed that this kinase activity was associated with two pro-

teins of about 43 and 45 kDa (Fig. 4b). These results suggested that p43 and p45 represent two isoforms of MAP-kinase (ERK2 and 1, respectively), and that both were activated following the LPA addition, although the tyrosine phosphorylation of ERK2 (p43) appeared dominant.

Our results showed that LPA, a bioactive phospholipid and strong mitogen, induces tyrosine phosphorylation of multiple proteins, two of which were identified as MAP-kinase and FAK. FAK, first identified as one of the major substrates of $p60^{src}$, is itself a tyrosine kinase present in focal adhesion and presumably involved in mitogenic signalling of the *src* product [15,16]. Its tyrosine phosphorylation and activation is induced by mitogenic peptides and by cell adhesion to matrix ligands [17–20]. On the other hand, tyrosine phosphorylation and activation of MAP-kinase is induced by various stimuli via both receptor tyrosine kinases and G-protein-coupled receptors, and is apparently mediated by two independent kinase cascades which converge at the MAP-kinase activator (MAP-kinase kinase). One of these cascades involving receptor tyrosine kinase, *ras* p21, *raf* kinase, and MAP-kinase kinase, has recently been clarified, whereas the other involving G-protein-coupled receptors and protein kinase C is poorly understood [21,22]. Van Corven et al. reported activation of $p21^{ras}$ by LPA and involvement of the Gi subfamily in this activation [9], which may account for LPA-induced activation of MAP-kinase. However, *ras*-transformed NIH3T3 cells show no increase in phosphorylation of FAK [23]. Our present study suggests that the LPA-evoked signal diverges to *ras*-dependent and -independent pathways. Questions raised by the present study are how tyrosine phosphorylation reactions for FAK, MAP-kinase and other proteins are linked in the LPA signalling pathway and are related to LPA-induced cell responses. LPA acts on a G-protein-coupled receptor and induces several responses in cells, including formation of stress fiber and focal adhesion [8]. This response is mediated by a small molecular weight GTP-binding protein, *rho*, which is involved in the organization of integrin-mediated cell adhesion [24,25]. In an effort to clarify the LPA pathway further, we are now analyzing the role of the *rho* protein in LPA-induced protein tyrosine phosphorylation.

Acknowledgements We thank Professor T. Kita for encouragement and support to N.K., and Miss Y. Kishimoto and K. Okuyama for technical and secretarial assistance, respectively. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (04253213, 04255103, 05271103, 05404020, 05670088) and grants from the Mitsubishi Foundation, the Senri Life Science Foundation, the Sagawa Cancer Research Foundation, and the Japanese Foundation on Metabolism and Diseases.

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