RESEARCH COMMUNICATION

G_i-mediated tyrosine phosphorylation of Grb2 (growth-factor-receptor-bound protein 2)-bound dynamin-II by lysophosphatidic acid

Onno KRANENBURG[1], Ingrid VERLAAN[1] and Wouter H. MOOLENAAR[2]

Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, The Netherlands

Lysophosphatidic acid (LPA) is the prototypic G-protein-coupled receptor agonist that activates the Ras-mitogen-activated protein (MAP) kinase cascade through pertussis toxin (PTX)-sensitive G_i and enhanced tyrosine kinase activity. We recently detected a 100 kDa protein (p100) that binds to the C-terminal SH3 domain of growth-factor-receptor-bound protein 2 (Grb2) and becomes tyrosine phosphorylated in a PTX-sensitive manner in LPA-treated Rat-1 cells [Kranenburg, Verlaan, Hordijk and Moolenaar (1997) EMBO J. **16**, 3097–3105]. Through glutathione S-transferase-Grb2 affinity purification and microsequencing, we have now identified p100 as dynamin-II, a GTPase that regulates clathrin-mediated endocytosis. We show that in Rat-1 cells, Grb2-bound dynamin-II is rapidly tyrosine phosphorylated in response to LPA in a PTX-sensitive manner. Thus, tyrosine phosphorylation of Grb2-bound dynamin-II may be a critical event in G_i-mediated activation of the Ras-MAP kinase cascade in fibroblasts.

INTRODUCTION

Activation of the Ras-mitogen-activated protein (MAP) kinase cascade is a critical response to growth factors acting through either receptor tyrosine kinases or G-protein-coupled receptors. Lysophosphatidic acid (LPA), a serum-borne lipid mitogen, serves as the prototypic G-protein-coupled receptor agonist that activates Ras through pertussis toxin (PTX)-sensitive G_i and intermediate tyrosine kinase activity [1,2]. Several candidate tyrosine kinases have been proposed to serve as intermediates between G_i and Ras-MAP kinase activation, including Src family kinases and the 'transactivated' epidermal growth factor receptor [3–6]. These kinases phosphorylate docking proteins, such as Shc or GAB1, thereby recruiting the growth-factor-receptor-bound protein 2 (Grb2) adaptor which directs the GDP/GTP exchanger Sos to Ras. However, the picture of how cell surface receptors activate the Ras-MAP kinase cascade is becoming increasingly complex as new participants in this signalling pathway are being identified (for review see [7]).

In a recent study, we examined in further detail how LPA may activate the Ras-MAP kinase cascade in fibroblasts by searching for candidate intermediates that are tyrosine phosphorylated in response to LPA in a PTX-sensitive manner [8]. We reported the detection of a 100 kDa tyrosine phosphorylated protein (p100) that preferentially binds to the C-terminal SH3 domain of Grb2 in LPA-stimulated Rat-1 cells. The available evidence suggests that the Grb2–p100 complex participates in linking G_i-coupled receptors to MAP kinase signalling [8]. However, the identity of p100 has been elusive to date.

In the present study, we set out to identify p100. Through Grb2 affinity purification we show that p100 is dynamin-II, a ubiquitously expressed GTPase that drives the formation of clathrin-coated endocytic vesicles (reviewed in [9]). We show that dynamin-II becomes tyrosine phosphorylated in response to LPA in Grb2 complexes. LPA-induced tyrosine phosphorylation of dynamin-II, like Ras-MAP kinase activation [8], is sensitive to both PTX and wortmannin. Our results suggest that tyrosine phosphorylation of dynamin-II plays a critical role in coupling activated LPA receptors at the cell surface to G_i-mediated Ras-MAP kinase signalling. Furthermore, the identification of dynamin-II as a substrate for G_i-regulated protein tyrosine kinase activity suggests that mitogenic signalling by LPA involves endocytic trafficking events.

EXPERIMENTAL

Cell culture

Rat-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 8 % (v/v) fetal calf serum and antibiotics. Confluent cell cultures were grown in serum-free medium for 3 days and were subsequently stimulated with agonists. Where indicated, cells were pretreated with PTX (List Biol. Labs. Inc.; 16 h, 200 ng/ml) or wortmannin (Sigma; 30 min, 1 μM).

Protein purification and sequencing

Thirty large dishes of Rat-1 cells (15 cm) were either stimulated with LPA (1 μM) or not, and cells were lysed in a buffer containing 0.1 % Triton X-100, 10 % (v/v) glycerol, 50 mM Tris (pH 7.4), 150 mM NaCl and 5 mM EDTA supplemented with protease inhibitors. Lysates were cleared by centrifugation (14000 rev./min; Eppendorff centrifuge) and were incubated with 50 μg of glutathione S-transferase (GST) immobilized on glutathione beads for 1 h. The supernatant was subsequently incubated with 50 μg of GST-Grb2 immobilized on glutathione beads for an additional 1 h. Beads were washed five times with lysis buffer, boiled in sample buffer and run out on long SDS/polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue, and the

Abbreviations used: Grb2, growth-factor-receptor-bound protein 2; GST, glutathione S-transferase; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; PTX, pertussis toxin.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail wmoolen@nki.nl).

100 kDa bands were cut out and sent for sequencing (Eurosequence, Groningen, The Netherlands). Four internal peptides were purified by HPLC and sequenced.

Immunoprecipitation and Western blotting

Cells were stimulated with LPA for 3 min and lysed in a buffer containing 1% Nonidet P40, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 50 mM Tris (pH 7.4) and protease inhibitors. Cell lysates were incubated with anti-dynamin antibodies (Transduction Laboratories, Lexington, KY, U.S.A.; #41) and Protein A beads for 2 h. The beads were washed, taken up in sample buffer and were run out on SDS/polyacrylamide gels. The gels were blotted onto nitrocellulose and were incubated with anti-phosphotyrosine antibody, followed by secondary rabbit anti-mouse-conjugated horseradish peroxidase (DAKO). Signals were detected using the enhanced chemiluminescence system (ECL; Amersham). The blots were stripped by incubation for 30 min at 50 °C in a buffer containing 2% SDS, 100 mM 2-mercaptoethanol and 50 mM Tris (pH 6.7). Blots were then washed in Tris-buffered saline/Tween and subsequently probed with anti-dymanin antibody.

Two-dimensional gel analysis

Cells were lysed and Grb2-associated proteins were isolated as above. The beads were washed five times in lysis buffer and were subsequently analysed by two-dimensional gel electrophoresis as described [10].

FPLC analysis

Cells were stimulated with 1 μM LPA for 3 min and Grb2-associated proteins were isolated as above. The beads were taken up in 50 μl FPLC buffer (20 mM Tris pH 7.4/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/0.02% Triton X-100) containing 0.1% SDS and were subsequently heated at 100 °C for 5 min. Samples were then diluted to 3 ml in FPLC buffer without SDS and were subsequently run on an FPLC monoQ column using an NaCl elution gradient (0–1 M). Fractions were collected and precipitated with 10% trichloroacetic acid. Precipitated samples were then taken up in lysis buffer and analysed by SDS/PAGE and subsequent Western blotting using anti-phosphotyrosine and anti-dynamin-II (Transduction Labs. #27) antibodies consecutively.

RESULTS AND DISCUSSION

Identification of dynamin-II as the major Grb2-binding protein in Rat-1 cells

We have previously shown that in Rat-1 cells, a 100 kDa Grb2-SH3 binding protein (p100) becomes tyrosine-phosphorylated in response to LPA or thrombin acting via G_i [8]. No p100 tyrosine phosphorylation was observed in response to endothelin, which signals through G_q and downstream phospholipase C, but fails to activate the G_i-Ras pathway in Rat-1 cells [2,8].

We set out to identify p100 through large-scale purification of Grb2-associated proteins from Rat-1 cell lysates. GST-Grb2 affinity purification followed by SDS/PAGE revealed one major Grb2-binding protein (Coomassie Blue staining; Figure 1). Microsequencing of tryptic peptides, purified by HPLC, identified this protein as dynamin-II (Table 1). Dynamin is a ubiquitous 100 kDa GTPase involved in endocytosis and known to bind Grb2-SH3. It is also the major Grb2-SH3 domain-binding protein in other cells (e.g. Ras-transformed NIH3T3 cells [10]).

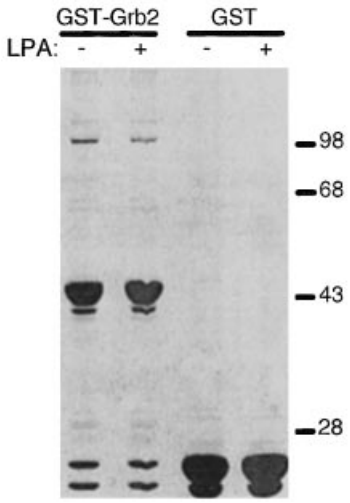

Figure 1 Purification of p100

Lysates from stimulated (3 min; 1 μM LPA) or unstimulated Rat-1 cells from 30 large dishes (15 cm) were pre-incubated with GST immobilized on glutathione beads. The supernatant was subsequently incubated with GST-Grb2. GST- and GST-Grb2-binding proteins were then separated on long SDS/polyacrylamide gels and were subsequently analysed by Coomassie staining. Molecular mass markers (kDa) are indicated on the right. The major Grb2-binding protein has an apparent molecular mass of 100 kDa.

Table 1 Internal peptide sequences

The 100 kDa protein (Figure 1) was cut from the gel and internal peptides were generated, purified by HPLC and sequenced. The amino acid sequences of the peptides, which are identical to sequences in rat dynamin-II, are shown.

Peptide	Sequence	Residues in dynamin-II
1	TLNQQLTNHI	280–289
2	EVEEYKNFRPDD	308–319
3	TGLFTPDLAF	400–409
4	EALNIIGDI	730–738

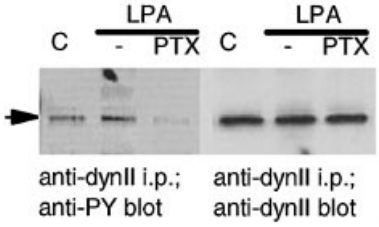

Figure 2 Dynamin-II tyrosine phosphorylation

Serum-starved Rat-1 cells were stimulated with LPA (1 μM; 3 min) in the presence or absence of PTX (200 ng/ml; 16 h pretreatment), as indicated. Cell lysates were prepared and dynamin-II was immuno-precipitated using anti-dynamin-II antibody. Dynamin tyrosine phosphorylation was then assayed by anti-phosphotyrosine blotting. After stripping (see the Experimental section), the blots were re-probed with anti-dynamin-II antibody to reveal immunoprecipitated dynamin-II. The arrow indicates the position of dynamin-II. C, control.

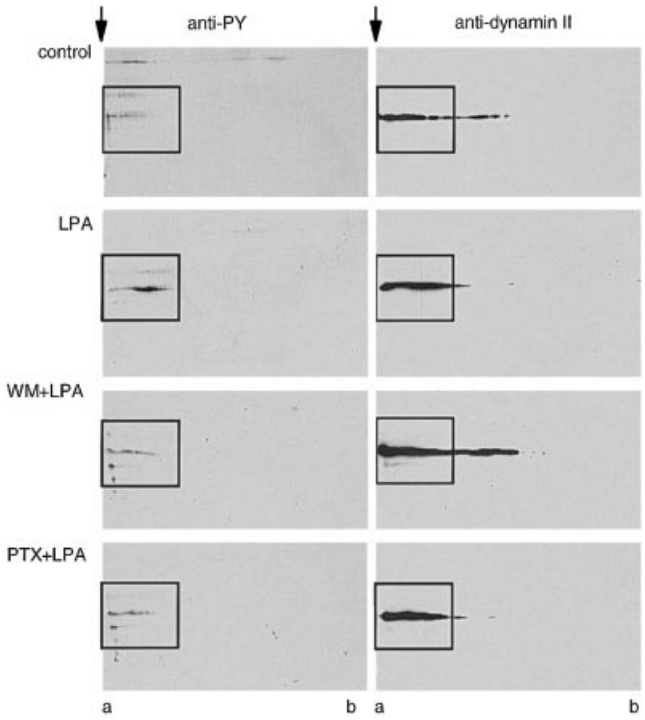

Figure 3 Two-dimensional gel analysis of tyrosine-phosphorylated dynamin-II in Grb2 complexes

Rat-1 cells were stimulated with LPA as in Figure 2. Where indicated, cells were pretreated with PTX or wortmannin (WM). Cell lysates were prepared and incubated with GST-Grb2. Grb2-bound proteins were isolated using glutathione-Sepharose and applied to a first dimension isoelectric focusing column. Columns were applied to SDS/polyacrylamide gels, which were run overnight (second dimension). The gels were blotted onto nitrocellulose and the blots were probed with anti-phosphotyrosine and anti-dynamin-II antibodies consecutively. Arrowheads indicate the loading positions; a, b: acidic and basic buffers respectively, during isoelectric focusing.

We also identified dynamin-II in anti-Grb2 immunoprecipitates from Rat-1 cells (results not shown), in support of the notion that dynamin constitutively associates with Grb2 in intact cells. In all experiments, Grb2-bound dynamin-II exactly co-migrated with tyrosine-phosphorylated p100 on long slab-gels (results not shown), suggesting that tyrosine-phosphorylated p100 is dynamin-II.

Tyrosine phosphorylation of Grb2-bound dynamin-II

To confirm that dynamin-II becomes tyrosine-phosphorylated in response to LPA, we used several antibodies against dynamin. Among the various anti-dynamin antibodies tested, one antibody (Transduction Laboratories #41) performed reasonably well in immune precipitations. Using this antibody, we found that stimulation of Rat-1 cells with LPA does indeed result in tyrosine phosphorylation of dynamin-II (Figure 2). LPA-induced tyrosine phosphorylation of dynamin-II, like that of p100 [8], is inhibited by PTX pretreatment, indicating that it is G_i-mediated (Figure 2).

We next examined the tyrosine phosphorylation state of dynamin-II in Grb2 complexes. Cells were stimulated with LPA, lysed and cell lysates were incubated with GST-Grb2. Grb2-bound proteins were resolved by two-dimensional gel electrophoresis, followed by Western blot analysis using anti-phosphotyrosine and anti-dynamin antibodies consecutively. Figure 3 shows that LPA induces tyrosine phosphorylation of a 100 kDa spot (in some gels visible as two distinct spots) that is recognized by anti-dynamin-II antibody. On longer exposures, we also observed tyrosine-phosphorylated p90 and p120 [8], but these run at a more basic pI and were not recognized by anti-dynamin antibody (not shown). Figure 3 further shows that LPA-induced tyrosine phosphorylation of Grb2-bound dynamin-II, like that of p100 [8], is sensitive to both PTX and wortmannin.

We noted that LPA causes an acidic pI shift in the migration pattern of dynamin, presumably as a result of phosphorylation (Figure 3). It can be seen that PTX blocks tyrosine phosphorylation of dynamin but not its pI shift, indicating that tyrosine phosphorylation cannot account for the pI shift observed and strongly suggesting that dynamin is phosphorylated on additional residues. Indeed, ^{32}P-labelling experiments revealed that dynamin-II is heavily phosphorylated in non-stimulated Rat-1 cells (results not shown). This makes it difficult to estimate the percentage of dynamin-II that becomes tyrosine phosphorylated after receptor stimulation. In our previous study [8], we have interpreted LPA-induced tyrosine phosphorylation of Grb2-bound p100 as complex formation. However, from the present results (Figures 1 and 3), we conclude that dynamin-II is constitutively bound to Grb2 and that LPA induces tyrosine phosphorylation of dynamin-II in a pre-existing complex with Grb2.

Finally, we separated Grb2-associated proteins by FPLC. Rat-1 cells were stimulated with LPA, Grb2-bound proteins were pulled down from the lysates and, after denaturation, the isolated proteins were applied to a monoQ column and separated by FPLC using an NaCl gradient. Figure 4 shows that the elution

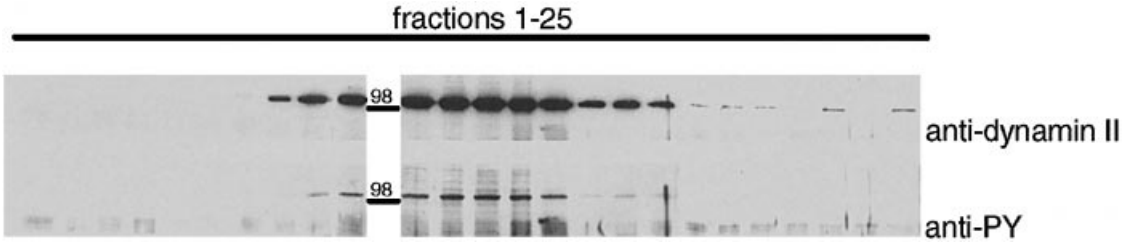

Figure 4 FPLC analysis of tyrosine phosphorylated dynamin-II in Grb2 complexes

Rat-1 cells were stimulated with LPA and cell lysates were incubated with GST-Grb2 as in Figure 3. Grb2-bound proteins were denatured by 5 min heating (100 °C) and the SDS was diluted out in FPLC buffer. MonoQ columns were run using a 0–1M NaCl gradient. The collected fractions were precipitated with trichloroacetic acid and loaded onto SDS/polyacrylamide gels. Western blot analysis using anti-phosphotyrosine and anti-dynamin antibodies shows that tyrosine-phosphorylated p100 and dynamin elute in the same fractions.

profiles of tyrosine-phosphorylated p100 and dynamin-II coincide, consistent with p100 being dynamin-II. Taken together, our results indicate that in quiescent Rat-1 cells, Grb2-bound dynamin-II becomes tyrosine phosphorylated in response to LPA through G_i.

Concluding remarks

The identification of p100 as dynamin-II, together with our previous findings [8], suggests that tyrosine phosphorylation of Grb2-bound dynamin-II plays a role in Ras-MAP kinase activation by LPA. The dynamin GTPase regulates membrane endocytosis by oligomerizing around the neck of endocytosing vesicles [12]. Concerted hydrolysis of GTP by the dynamin oligomers then provides the force for fission of the vesicle from the membrane [13]. It is becoming increasingly clear that clathrin-based endocytosis plays an important role in the regulation of signalling pathways, both positively and negatively [14–16]. Recent reports have shown that MAP kinase activation is abrogated when endocytosis is blocked using a mutant form of dynamin that has low affinity for nucleotides and reduced GTPase activity [14–16]. Yet another link between mitogenic signalling and endocytosis is the finding that Grb2 is essential for receptor internalization [17]. Furthermore, proteins involved in endocytosis have been found mutated in human cancers, possibly causing the dysregulation of signalling pathways [18]. Conversely, various signalling molecules have been implicated in receptor endocytosis [17,19–23]. In particular, tyrosine kinase activity is required for both MAP kinase activation and receptor internalization [19,20].

Dynamin function is regulated by protein–protein interactions, by lipid binding and by phosphorylation (reviewed in [9]). Dynamin can be phosphorylated on both serine/threonine and tyrosine residues [9]. While protein kinase C-induced phosphorylation of dynamin stimulates its GTPase activity [24], the functional consequence of tyrosine phosphorylation is unknown. In cells overexpressing insulin receptors, dynamin is rapidly tyrosine phosphorylated by insulin [25]. Similarly, tyrosine phosphorylation of dynamin has been observed in monocytes stimulated with colony-stimulating factor [26]. It has been reported recently that activation of β_2-adrenergic receptors leads to c-Src-mediated tyrosine phosphorylation of dynamin in transiently transfected HEK293 cells, and that this event is required for MAP kinase signalling [27]. We are currently examining the consequences of dynamin tyrosine phosphorylation for both MAP kinase activation and clathrin-mediated endocytosis. Whatever the precise function of its tyrosine phophorylation, the identification of dynamin-II as a substrate for G_i-regulated tyrosine kinase activity strengthens the notion that receptor-mediated endocytosis and Ras-MAP kinase signalling are interconnected events.

Received 1 February 1999; accepted 9 February 1999

We thank P. Spee for expert assistance with two-dimensional gel analysis. This work was supported by the Dutch Cancer Society.

REFERENCES

1 Moolenaar, W. H., Kranenburg, O., Postma, F. R. and Zondag, G. C. M. (1997) Curr. Opin. Cell Biol. **9**, 168–173
2 van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L. and Moolenaar, W. H. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 1257–1261
3 Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J. and Lefkowitz, R. J. (1996) J. Biol. Chem. **271**, 19443–19450
4 Daub, H., Weiss, F. U., Wallasch, C. and Ullrich, A. (1996) Nature (London) **379**, 557–560
5 Wan, Y., Bence, K., Hata, A., Kurosaki, T., Veillette, A. and Huang, X. Y. (1997) J. Biol. Chem. **272**, 17209–17215
6 Wan, Y., Kurosaki, T. and Huang, X. Y. (1996) Nature (London) **380**, 541–544
7 Sternberg, P. W. and Alberola-Ila, J. (1998) Cell **95**, 447–450
8 Kranenburg, O., Verlaan, I., Hordijk, P. L. and Moolenaar, W. H. (1997) EMBO J. **16**, 3097–3105
9 McClure, S. J. and Robinson, P.J (1996) Mol. Membr. Biol. **13**, 189–215
10 O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H. (1977) Cell **12**, 1133–1141
11 Yoon, S. Y., Koh, W. S., Lee, M. K., Park, Y. M. and Han, M. Y. (1997) Biochem. Biophys. Res. Commun. **234**, 539–543
12 Hinshaw, J. E. and Schmid, S. L. (1995) Nature (London) **374**, 190–192
13 Sweitzer, S. M. and Hinshaw, J. E. (1998) Cell **93**, 1021–1029
14 Vieira, A. V., Lamaze, C. and Schmid, S. L. (1996) Science **274**, 2086–2089
15 Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G. and Lefkowitz, R. J. (1998) J. Biol. Chem. **273**, 685–688
16 Ceresa, B. P., Kao, A. W., Santeler, S. R. and Pessin, J. E. (1998) Mol. Cell Biol. **18**, 3862–3870
17 Wang, Z. and Moran, M. F. (1996) Science **272**, 1935–1939
18 Floyd, S. and Camilli, P.D (1998) Trends Cell Biol. **8**, 299–301
19 Glenney, Jr., J. R., Chen, W. S., Lazar, C. S., Walton, G. M., Zokas, L. M., Rosenfeld, M. G. and Gill, G. N. (1988) Cell **52**, 675–684
20 Lamaze, C. and Schmid, S. L. (1995) J. Cell Biol. **129**, 47–54
21 Lohi, O. and Lehto, V. P. (1998) FEBS Lett. **432**, 225–227
22 Okabayashi, Y., Sugimoto, Y., Totty, N. F., Hsuan, J., Kido, Y., Sakaguchi, K., Gout, I., Waterfield, M. D. and Kasuga, M. (1996) J. Biol. Chem. **271**, 5265–5269
23 Ware, M. F., Tice, D. A., Parsons, S. J. and Lauffenburger, D. A. (1997) J. Biol. Chem. **272**, 30185–30190
24 Robinson, P. J., Sontag, J. M., Liu, J. P., Fykse, E. M., Slaughter, C., McMahon, H. and Sudhof, T. C. (1993) Nature (London) **365**, 163–166
25 Baron, V., Alengrin, F. and Van Obberghen, E. (1998) Endocrinology **139**, 3034–3037
26 Kharbanda, S., Saleem, A., Yuan, Z., Emoto, Y., Prasad, K. V. and Kufe, D. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 6132–6136
27 Ahn, S., Maudsley, S., Luttrell, L. M., Lefkowitz, R. J. and Daaka, Y. (1999) J. Biol. Chem. **274**, 1185–1188