Lysophosphatidic acid-induced activation of protein Ser/Thr kinases in cultured rat 3Y1 fibroblasts
Possible involvement in *rho p21*-mediated signalling

Naokazu Kumagai, Narito Morii, Toshimasa Ishizaki, Naoki Watanabe, Kazuko Fujisawa, Yuji Saito, Shuh Narumiya*

Department of Pharmacology, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606, Japan

Received 24 March 1995; revised version received 24 April 1995

Abstract Renaturation kinase assay was used to detect protein kinases activated by lysophosphatidic acid (LPA) in cultured rat 3Y1 fibroblasts. LPA activated several Ser/Thr protein kinases with apparent molecular weights of 145K, 85K, 64–65K (a doublet), and 60K (each named *p145, p85, p64165* and *p60*, respectively) in addition to *p43* mitogen activated protein (MAP)-kinase. Experiments using pertussis toxin and botulinum C3 exoenzyme showed that *p145, p85,* and *p64165* kinases were activated by a pertussis toxin-insensitive *rho p21*-dependent pathway and that the activation of MAP-kinase was mediated by both the pertussis toxin-sensitive *rho p21*-independent and the pertussis toxin-insensitive *rho p21*-dependent pathways.

Key words: rho p21; Lysophosphatidic acid; Protein Ser/Thr kinase; Botulinum C3 exoenzyme; Pertussis toxin; Rat 3Y1 fibroblast

1. Introduction

Lysophosphatidic acid (LPA) is a platelet derived serum factor that evokes a wide range of biological effects, including the stimulation of fibroblast proliferation, platelet aggregation, cell motility, tumor cell invasion and neurite retraction [1]. LPA appears to activate its own receptor(s) coupling to both PTX-sensitive and insensitive G-proteins. One characteristic response found in fibroblasts is the induction of focal adhesion and stress fiber formation, which is mediated by a small molecular weight GTP-binding protein, *rho p21* [2]. *rho p21* is a member of the *ras* superfamily of small GTPases. The functions of this protein have been examined by the use of *botulinum* C3 exoenzyme, which specifically ADP-ribosylates and inactivates this protein [3]. Studies using this exoenzyme revealed that *rho p21* mediates a number of cellular processes, such as stimulus-evoked cell adhesion in fibroblasts [2], platelets [4] and lymphocytes [5], cell motility in leukocytes [6] and fibroblasts [7], G1 to S phase progression of the cell cycle [8], neurite retraction stimulated by LPA and thrombin [9], the regulation of smooth muscle contraction [10] and cytokinesis during cell division [11,12]. We and others have previously demonstrated that LPA induced the tyrosine phosphorylation of a variety of proteins in cultured cells [13,14]. We further showed that some of these, e.g. the phosphorylation of *p125* FAK, were significantly attenuated by the C3 exoenzyme-catalyzed ADP-ribosylation of *rho p21*, suggesting that *rho p21* served as a transducer linking LPA signalling to the tyrosine phosphorylation [15]. A similar finding has recently been obtained in protein tyrosine phosphorylation induced by bombesin and endothelin in Swiss 3T3 cells [16]. However, the signalling pathway linking *rho p21* to tyrosine phosphorylation has remained elusive. In this study, using a renaturation kinase assay [17], we have analyzed protein kinase species activated by LPA in cultured cells, and examined their sites of activation in the signalling pathway. Our results showed that several protein serine/threonine kinases are activated by LPA and that some of them appear to be downstream of *rho p21* in the signal transduction pathway.

*Corresponding author. Fax: (81) (75) 753-4693.

Abbreviations: C3, exoenzyme; C3, ADP-ribosyltransferase from *Clostridium botulinum*; ERK, extracellular signal-regulated kinase; G-protein, a heterotrimeric GTP-binding protein; LPA, lysophosphatidic acid; MAP-kinase, mitogen-activated protein kinase; *p125* FAK, focal adhesion kinase of 125 KDa; PBS, phosphate-buffered saline; PLC, phospholipase C; PKC, protein kinase C; PTX, pertussis toxin; *rho p21*, the small GTP-binding protein of the *rho* gene product.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (3,000 Ci/mmol) and [α-^{32}P]NAD (800 Ci/mmol) were purchased from Dupont New England Nuclear. LPA (1-oleoyl-*sn*-glycero-3-phosphate), phosphoserine, phosphothreonine and phosphotyrosine were obtained from Sigma. LPA was dissolved in 20% ethyl alcohol to 20 mM as a stock solution, stored at −80°C and then diluted with the serum-free culture medium [13] to 20 μM before use. PTX (islet activating protein from *Bordetella pertussis*) was obtained from Funakoshi Co. Ltd., Tokyo. Lipofectamine was obtained from Life Technologies. Recombinant C3 exoenzyme was prepared as previously described [15]. All other chemicals used were of reagent grade.

2.2. Cell culture

Rat 3Y1 fibroblasts were cultured until near confluency in DMEM containing 10% fetal calf serum as described previously [13], and subjected to either C3 exoenzyme or PTX treatment. The cells were incubated with or without 10 μg/ml C3 exoenzyme first in complete medium for 48 h, and then in the serum-free medium for 12 h, as described [15]. To facilitate exoenzyme incorporation, 40 μg of the exoenzyme was mixed with 40 μl of lipofectamine and added to the cells during the latter incubation. Alternatively, they were incubated with 20 ng/ml of PTX in the serum-free medium for 12 h prior to the LPA stimulation. The cells were then incubated with 2 μM LPA for the indicated times at 37°C in a CO_2 incubator. LPA induced cell response from 20 nM in a concentration-dependent manner, which reached the maximum at 2 μM [13]. After incubation, the cells were rinsed with 4 ml per well of ice-cold PBS, and then lysed on ice for 10 min with 400 μl of RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 100 kU/ml aprotinin, 1 mM Na_3VO_4, 10 μM Na_3MoO_4 and 10 mM NaF. The lysates were centrifuged at 12,000 × *g* for 15 min, and the supernatants were used for the subsequent analysis.

SSDI 0014-5793(95)00478-5

2.3. Renaturation kinase assay

The supernatants (30 μg protein each) were subjected to SDS-PAGE on an 8% polyacrylamide gel, and the separated proteins were transferred to PVDF membranes (Millipore) as described previously [13]. Denaturation and renaturation of transferred proteins were performed essentially as described by Ferrell and Martin [17]. After renaturation, the membrane was blocked with 5% BSA in 30 mM Tris-HCl, pH 7.5, at room temperature for 1 h, rinsed and incubated with 20 nM (50 μCi/ml) [^{32}P]ATP for 30 min at 30°C in 3 ml of the incubation solution (30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$). The reaction was terminated by adding 100 ml of ice-cold 30 mM Tris-HCl, pH 7.5 (the rinsing solution). The membrane was then rinsed at room temperature twice with the rinsing solution, once with a solution containing 0.05% Tween-20, and then again with the rinsing solution for 10 min per rinse. The membrane was then incubated in 1 N KOH for 10 min at room temperature, then incubated twice in 1.7 M acetic acid for 5 min each and finally rinsed with distilled water several times for 10 min. The membrane was then dried and subjected to autoradiography.

In this assay, ^{32}P incorporation into several bands (see below) increased linearly for 60 min using cell lysates in the range of 5–80 μg protein/lane. ^{32}P incorporation also increased with increasing concentrations of [^{32}P]ATP, and saturated at 100–300 nM with essentially the same findings. In some experiments, the cell lysates were subjected to immunoprecipitation with the anti-ERK-2 antibody 2Y [13,18], and the immunoprecipitates were also analyzed by this assay.

2.4. Phosphoamino acid analysis

[^{32}P]Phosphorylated bands on the PVDF membranes were excised and were subjected to hydrolysis in 6 N HCl for 70 min at 105°C as previously described [19]. The hydrolysates were lyophylized and subjected to electrophoresis on cellulose thin layer plates (100 μm thickness) as described [20]. The radioactive spots were visualized by autoradiography and/or by a Bioimage analyzer BAS2000 (Fujix, Tokyo).

2.5. ADP-ribosylation

After incubation with PTX, C3 exoenzyme or vehicle, the cells were rinsed twice with PBS pre-warmed to 37°C, and then incubated with 1.0% trypsin and 0.02% EDTA in PBS. The dissociated cells were collected by centrifugation at 800 × *g* for 3 min, suspended in 100 μl of the ADP-ribosylation buffer containing [^{32}P]NAD plus 50 ng activated PTX or 100 ng C3 exoenzyme, and then sonicated. The incubation and quantification were carried out as previously described [21,22]. The amount of protein was determined by the method of Bradford [23].

3. Results and discussion

We used a renaturation kinase assay to examine the protein kinases activated in response to LPA in cultured rat 3Y1 cells. This method exploits the fact that the enzymatic activity of

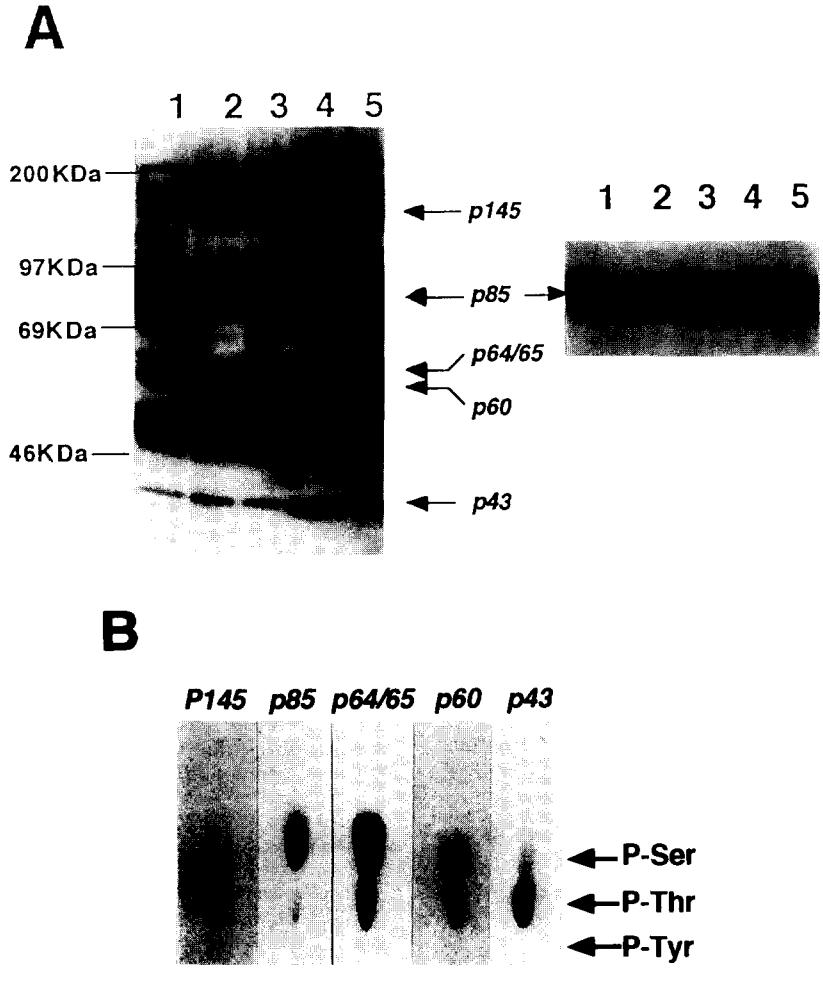

Fig. 1. Renaturation kinase analysis (A) and phosphoamino acid analysis (B) of LPA-activated kinases in rat 3Y1 fibroblasts. (A) Cells were incubated with 2 μM LPA for 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 5 (lane 4) and 10 (lane 5) min. Cell lysates were prepared and subjected to the renaturation kinase analysis as described under section 2. A typical result from at least five experiments is shown. Shown on the right is an autoradiogram of the same membrane obtained by shorter exposure, showing the activation of *p85* kinase. (B) [^{32}P]Phosphorylated bands (*p145*, *p85*, *p64/65*, *p60* and *p43*) observed in the renaturation kinase analysis as above of cell lysates at 5 min of LPA stimulation were excised and phosphorylated amino acid of each band was analyzed. P-Ser, P-Thr and P-Tyr denote the electrophoretic positions of phosphoserine, phosphothreonine and phosphotyrosine, respectively.

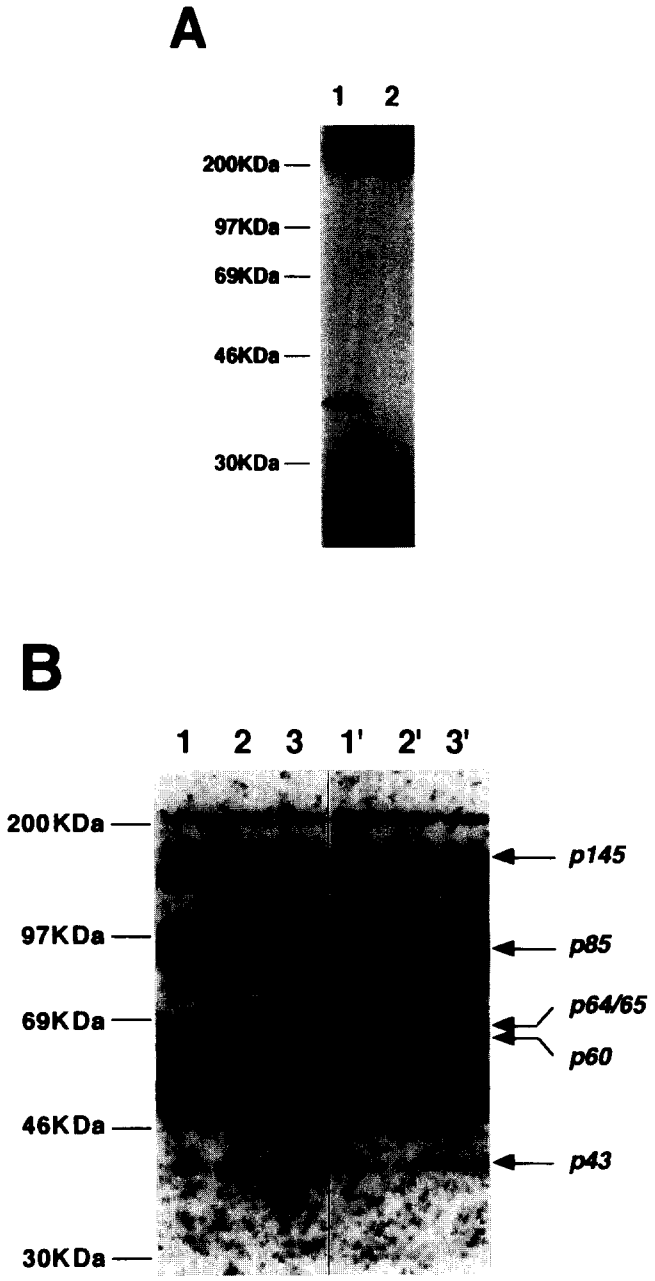

Fig. 2. Effects of PTX treatment on the LPA-induced kinase activation. (A) [^{32}P]ADP-ribosylation of a PTX substrate in lysates from control (lane 1) and PTX-treated (lane 2) cells. (B) Renaturation kinase analysis of control (lanes 1–3) and PTX-treated (lanes 1′–3′) cells stimulated by LPA for 0 min (lanes 1 and 1′), 1 min (lanes 2 and 2′) and 5 min (lanes 3 and 3′).

some protein kinases can be renatured after SDS-PAGE and blotting onto the membrane [24] and the fact that protein kinases are often activated by covalent modification and show enhanced autophosphorylation. After serum starvation for 12 h, 3Y1 cells were stimulated with 2 μM LPA for various times, and lysates were prepared and subjected to analysis. As shown in Fig. 1A, LPA increased the activity of several kinases, with molecular weights of 145K, 85K, 64–65K (a doublet), 60K and 43K, which were tentatively named *p145, p85, p64/65, p60* and *p43* kinase, respectively. The time course and extent of activation appeared to be different for each of these proteins. For example, *p145* was activated rapidly, reached a peak at 0.5–1 min and maintained the higher activity than baseline through incubation. On the other hand, *p85* was activated throughout the entire 10 min incubation. The activation of *p64/65* and *p60* was transient, with a peak at 1 min. Transient activation was also found for *p43*, which peaked at 5 min but then declined. This time course for *p43* was reminiscent of the LPA-induced activation of MAP-kinase in Swiss 3T3 cells [13,15]. We, therefore, immunoprecipitated the cell lysates with an anti-ERK2 antibody, and performed the renaturation kinase assay on the precipitates. This procedure specifically precipitated *p43*, suggesting that it is the MAP-kinase, ERK-2 (data not shown).

To characterize the reactions catalyzed by these proteins, we excised the [^{32}P]phosphorylated bands and subjected them to phosphoamino acid analysis. As shown in Fig. 1B, all of the bands analyzed yielded either phosphoserine or phosphothreonine but not phosphotyrosine, as the major [^{32}P]phosphoamino acid. Phosphothreonine was found in *p145*, and phosphoserine was detected in *p85* and both in *p64/65* and *p60*. *p43* MAP-kinase showed the increased phosphorylation of threonine in this analysis. These phosphorylations appeared to be due to autophosphorylation, because the omission of BSA from the solution did not affect the phosphorylation pattern. These results indicate that these kinases are protein serine/threonine kinases. This is consistent with a previous study reporting that this method preferentially detects Ser/Thr kinases [17].

Since LPA activates multiple signalling pathways and one of them is mediated by G_i, [1], PTX was used to examine the involvement of this G-protein in the activation of the above kinases. As shown in Fig. 2A, the incubation of the cells with 20 ng/ml PTX overnight completely ADP-ribosylated the endogenous G_i in the cells. Under these conditions, kinase activation as determined by the renaturation assay remained unaffected except for *p43* MAP-kinase (Fig. 2B), suggesting that the activation of MAP-kinase occurs at least in part through the G_i pathway. These results are consitent with the previous finding that LPA activated MAP-kinase mostly via G_i [14,25].

We next used botulinum C3 exoenzyme and examined the involvement of *rho p21*, because *rho p21* functions in the other pathway of LPA signalling as a transducer between the PTX-insensitive G-protein and cell adhesion [1,2,15]. As shown in Fig. 3A, the incubation of the cells with 10 μg/ml of exoenzyme induced over 80% ADP-ribosylation of the *rho p21* in the cells. This resulted in a significant attenuation of the activation of *p145, p85* and *p64/65* kinases. The activation of *p43* MAP-kinase was partially attenuated at 5 min, whereas *p60* kinase was only slightly affected (Fig. 3B).

Thus, we have demonstrated that several protein Ser/Thr kinases are activated in LPA signalling and their activation was interferred by inactivation of G_i and *rho p21*. Fig. 4 proposes a model for LPA-induced signal transduction pathways consistent with the previous studies and the present findings. LPA evokes a variety of biological actions by acting on its cognate receptor, coupling to both PTX-sensitive and insensitive G-proteins [1]. The pathway mediated by PTX-insensitive G-protein(s) involves PLC and PKC activation. *rho p21* appears to be activated in this pathway apparently independently of PKC activation [25,26]. Three Ser/Thr kinases (*p145*, *p85*, and *p64/65*) are activated downstream of *rho p21*, which also mediates protein tyrosine phosphorylation of *p125* FAK and *p72* paxillin

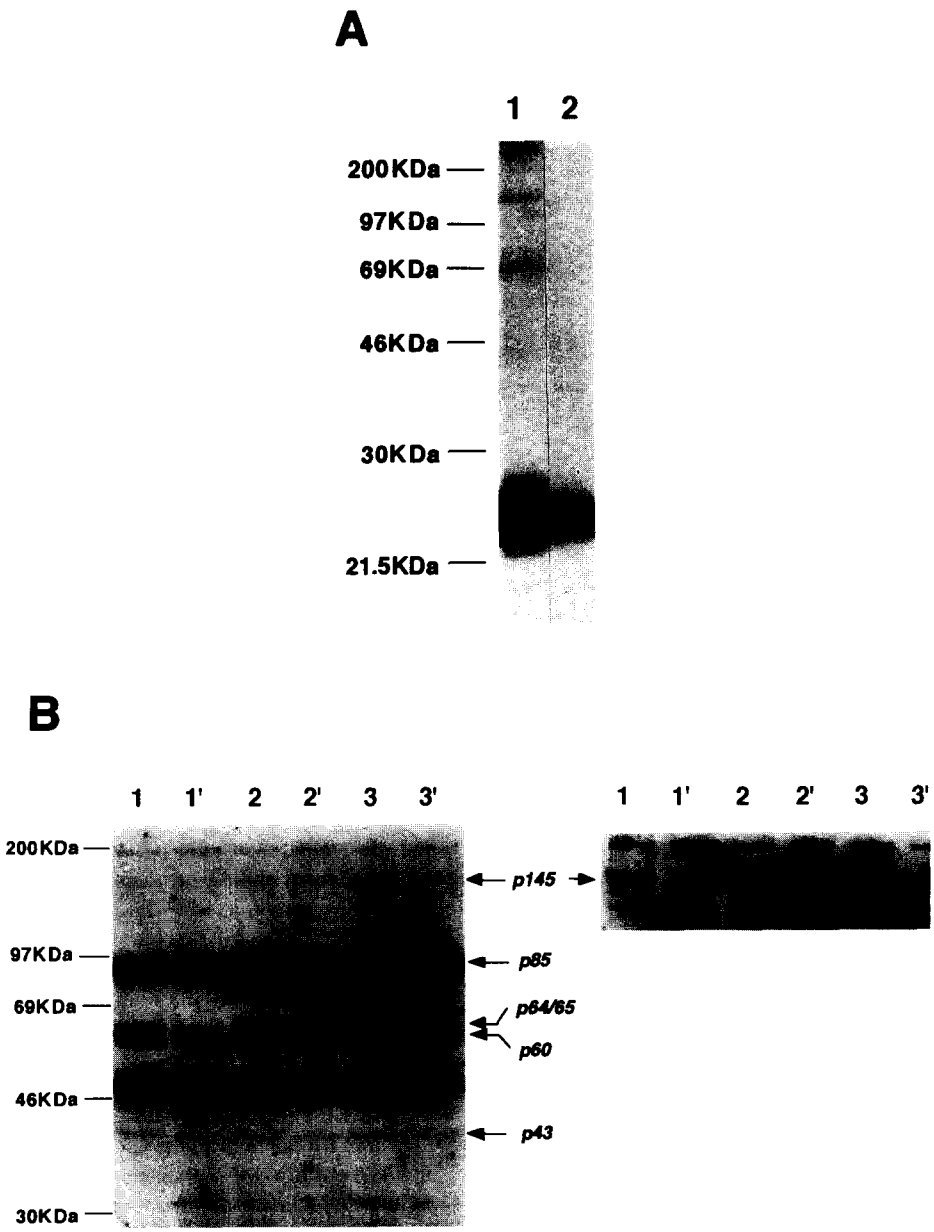

Fig. 3. Effects of C3 exoenzyme treatment on the LPA-induced kinase activation. (A) [^{32}P]ADP-ribosylation of *rho p21* in control and C3 exoenzyme-treated cells (lane 1 and 2, respectively). (B) Renaturation kinase analysis of control (lanes 1–3) and C3 exoenzyme-treated (lanes 1′–3′) cells stimulated by LPA for O min (lanes 1 and 1′), 1 min (lanes 2 and 2′) and 5 min (lanes 3 and 3′). The control cells received the same amount of lipofectamine. Shown in the right is an autoradiogram obtained by longer exposure of the same membrane, showing the inhibition of the *p145* kinase activation by C3 exoenzyme treatment.

[15]. (The p72 tyrosine-phosphorylated in response to LPA in a *rho p21*-dependent manner in Swiss 3T3 cells [15] was identified as paxillin by immunoprecipitation and immunoblot with anti-paxillin antibody (Kumagai, N., Morii, N. and Narumiya, S., unpublished observation)). This pathway leads to focal adhesion, stress fiber formation and activation of PI-3-kinase [15,16,25]. The PTX-sensitive pathway activates *ras p21*, which in turn activates a kinase cascade involving *raf* kinase, MEK and MAP-kinase, leading to cell proliferation [27,28]. MAP-kinase is also activated by the *rho p21*-dependent pathway, which contributes to the late phase of its activation [15]. These results strongly indicate that *rho p21* directly or indirectly activates the Ser/Thr kinases, which may form a kinase cascade further downstream. It has already been reported that the *rho p21*-related proteins *rac* and *CDC42 p21*s, but not *rho p21* itself, activate the Ser/Thr protein kinase PAK and the tyrosine kinase ACK [29,30]. Hence, *rho p21* may activate analogous kinases. The activation of the Ser/Thr kinases by *rho p21* may be linked to *p125* FAK activation, because FAK undergoes serine as well as tyrosine phosphorylation upon cell stimulation [31]. It is also possible that the above kinases function in the signal transduction of integrin-mediated cell adhesion, because *rho p21* activates integrin to induce cell adhesion to the substratum [2,4,5]. Recently, Chen et al. [32] reported that the integrin-mediated cell adhesion to the substratum can cause the activation of MAP-kinase. It is interesting to note that MAP-kinase

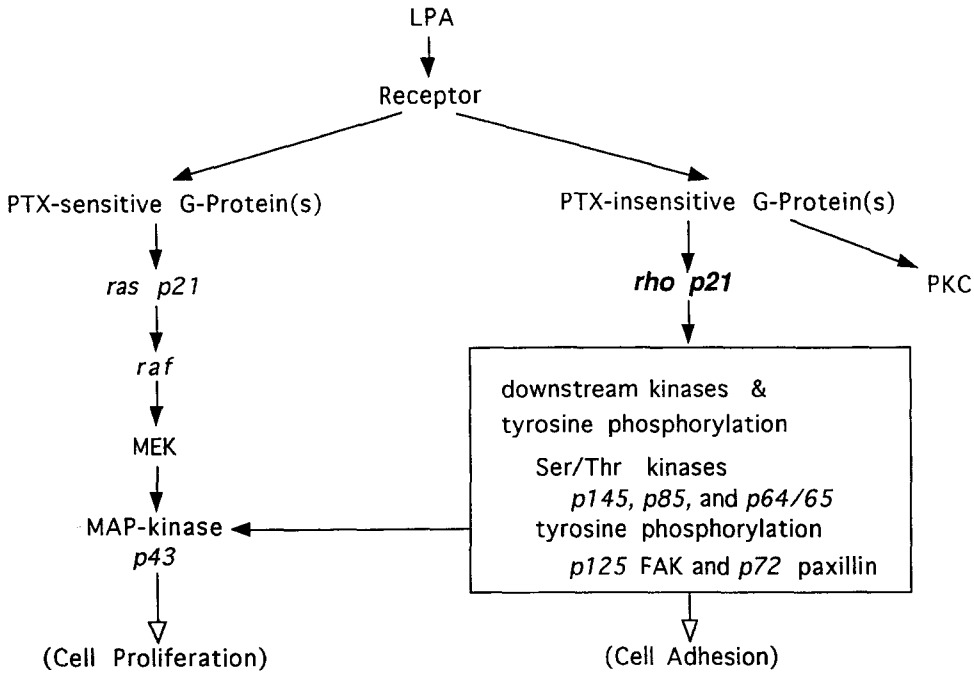

Fig. 4. A model of *rho p21* action in the LPA signalling pathways.

activation is mediated by both *ras p21* and *rho p21*-dependent pathways, and that the *rho p21*-dependent pathway contributes to the late phase of MAP-kinase activation (this study, and [15]). Finally, the findings obtained in the present study may not be limited to 3Y1 fibroblasts and LPA signalling, but may be applicable to similar transduction pathways in other types of cells. We have already obtained the essentially identical results in cultured Swiss 3T3 cells (data not shown). Furthermore, Ferrell and Martin [33] used the same method and found that a group of Ser/Thr kinases were activated in response to thrombin in platelets, several of which appear to overlap with the kinases found here. In summary, using the renaturation kinase assay, we have identified several Ser/Thr kinases activated in fibroblasts in response to LPA and found that some of them are mediated by *rho p21* in the cell. This is the first report showing the link between *rho p21* and Ser/Thr kinases, and should facilitate identification of the thus far unknown signal transduction of this small molecular weight GTP-binding protein.

Acknowledgements: We thank Drs. T. Kinashi and H. Sabe for their valuable advices, and Dr. T. Kimura of the Faculty of Technology, Kyoto University, for his help in the phosphoamino acid analysis. We also thank Y. Kishimoto and K. Okuyama for their assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (05271103, 06281231, 06275206, 06670107), and by grants from the Senri Life Science Foundation, the Human Frontier Science Program and the Naito Memorial Foundation.

References

[1] Moolenaar, W.H. (1994) Trends Cell Biol. 4, 213–219.
[2] Ridley, A.J. and Hall, A. (1992) Cell 70, 389–399.
[3] Narumiya, S. and Morii, N. (1993) Cell. Signalling 5, 9–19.
[4] Morii, N., Teruuchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. and Narumiya, S. (1992) J. Biol. Chem. 267, 20921–20926.
[5] Tominaga, T., Sugie, K., Hirata, M., Morii, N., Fukata, J., Uchida, A., Imura, H. and Narumiya, S. (1993) J. Cell Biol., 120, 1529–1537.
[6] Stasia, M.-J., Jouan, A., Bourmeyster, N., Bouquet, P. and Vignais, P.V. (1991) Biochem. Biophys. Res. Commun. 180, 615–622.
[7] Nishiyama, T., Sasaki, T., Takaishi, K., Kato, M., Yaku, H., Araki, K., Matsuura, Y. and Takai, Y. (1994) Mol. Cell. Biol. 14, 2447–2456.
[8] Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. (1993) Oncogene 8, 1449–1455.
[9] Jalink, K., van Corven, E.J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W.H. (1994) J. Cell Biol. 126, 801–810.
[10] Hirata, K., Kikuchi, A., Sasaki, T., Kuroda, S., Kaibuchi, K., Matsuura, Y., Seki, H., Saida, K. and Takai, Y. (1992) J. Biol. Chem. 267, 20921–20926.
[11] Mabuchi, I., Hamaguchi, Y., Fujimoto, H., Morii, N., Mishima, M. and Narumiya, S. (1993) Zygotes 1, 325–331.
[12] Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. and Takai, Y. (1993) J. Cell Biol. 120, 1187–1195.
[13] Kumagai, K., Morii, N., Fujisawa, K., Yoshimasa, T., Nakao, K. and Narumiya, S. (1993) FEBS Lett. 329, 273–276.
[14] van Corven, E.J., Hordijk, P.L., Medema, R.H., Bos, J.L. and Moolenaar, W.H. (1993) Proc. Natl. Acad. Sci. USA 90, 1257–1261.
[15] Kumagai, K., Morii, N., Fujisawa, K., Nemoto, Y. and Narumiya, S. (1993) J. Biol. Chem. 268, 24535–24538.
[16] Rankin, S., Morii, N., Narumiya, S. and Rozengurt, E. (1994) FEBS Lett. 354, 315–319.
[17] Ferrell Jr., J.E. and Martin, G.S. (1991) Methods Enzymol. 200, 430–435.
[18] Yoshimasa, T., Nakao, K., Suga, S., Kishimoto, I., Kiso, Y. and Imura, H. (1992) FEBS Lett. 311, 195–198.
[19] Kamps, M.P. (1991) Methods Enzymol., 201, 21–27.
[20] Duclos, B., Marcandier, S. and Cozzone, A.J. (1991) Methods Enzymol. 201, 10–21.
[21] Katada, T., Oinuma, M. and Ui, M. (1986) J. Biol. Chem. 261, 8182–8191.
[22] Morii, N. and Narumiya, S. (1994) Methods Enzymol. (in press).
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Celenza, J.L. and Carlson, M. (1991) Methods Enzymol. 200, 423–430.

[25] Ridley, A.J. and Hall, A. (1994) EMBO J. 13, 2600–2610.
[26] Seufferlein, T. and Rosengurt, E. (1994) J. Biol. Chem. 269, 9345–9351.
[27] Howe, L.R. and Marshall, C.J. (1993) J. Biol. Chem. 268, 20717–20720.
[28] Hodijk, P.L., Verlaan, I., van Corven, E.J. and Moolenaar, W.H. (1994) J. Biol. Chem. 269, 645–651.
[29] Manser, E., Leung, T., Salihuddin, H., Zhao, Z. and Lim, L. (1994) Nature 367, 40–46.
[30] Manser, E., Leung, T., Salihuddin, H., Tan, L. and Lim, L. (1993) Nature 363, 364–367.
[31] Hatai, M., Hashi, H., Mogi, A., Soga, H., Yokota, J. and Yaoi, Y. (1994) FEBS Lett. 350, 113–116.
[32] Chen, Q., Kinch, M.S., Lin, T.H., Burridge, K. and Juliano, R.L. (1994) J. Biol. Chem. 269, 26602–26605.
[33] Ferrell Jr., J.E. and Martin, G.S. (1989) J. Biol. Chem. 264, 20723–20729.