

# Physical and Functional Interactions of the Lysophosphatidic Acid Receptors with PDZ Domain-containing Rho Guanine Nucleotide Exchange Factors (RhoGEFs)\*

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**Lysophosphatidic acid (LPA) is a serum-derived phospholipid that induces a variety of biological responses in various cells via heterotrimeric G protein-coupled receptors (GPCRs) including LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub>. LPA-induced cytoskeletal changes are mediated by Rho family small GTPases, such as RhoA, Rac1, and Cdc42. One of these small GTPases, RhoA, may be activated via G $\alpha_{12/13}$ -linked Rho-specific guanine nucleotide exchange factors (RhoGEFs) under LPA stimulation although the detailed mechanisms are poorly understood. Here, we show that the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> but not LPA<sub>3</sub> interact with the PDZ domains of PDZ domain-containing RhoGEFs, PDZ-RhoGEF, and LARG, which are comprised of PDZ, RGS, Dbl homology (DH), and pleckstrin homology (PH) domains. In LPA<sub>1</sub>- and LPA<sub>2</sub>-transfected HEK293 cells, LPA-induced RhoA activation was observed although the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> mutants, which failed to interact with the PDZ domains, did not cause LPA-induced RhoA activation. Furthermore, overexpression of the PDZ domains of PDZ domain-containing RhoGEFs served as dominant negative mutants for LPA-induced RhoA activation. Taken together, these results indicate that formation of the LPA receptor/PDZ domain-containing RhoGEF complex plays a pivotal role in LPA-induced RhoA activation.**

Lysophosphatidic acid (LPA)<sup>1</sup> is a serum-derived phospholipid that induces a variety of biological responses in various cells (1–5). LPA is also the prototypic G protein-coupled receptor (GPCR) ligand that activates MAP kinase, phospholipase C, and small GTPases, etc., via heterotrimeric G proteins (1–5). Three distinct G protein-coupled receptors for

LPA have been identified, termed LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> (previously Edg2, Edg4, and Edg7, respectively). LPA signals induce actin rearrangements via the Rho family GTPase, RhoA, Rac1, and Cdc42 (1, 5–6). Rho family GTPases have GDP-bound inactive and GTP-bound active forms, the cycle of which is regulated by Rho guanine nucleotide exchange factors (RhoGEFs) that stimulate the exchange of GDP for GTP (7). Members of the RhoGEF family have a Dbl homology (DH) domain that catalyzes the exchange reaction and a pleckstrin homology (PH) domain immediately C-terminal to the DH domain (8). The PH domain is responsible for both subcellular localization and modulation of DH domain function (8). Recently, it has been recognized that the G<sub>12/13</sub> family mediates signaling from the LPA receptor to RhoA and that RhoGEFs containing regulators of G protein signaling (RGS) domains are involved in these processes (9–10). RGS domain-containing RhoGEFs have been described p115-RhoGEF, PDZ-RhoGEF, and leukemia-associated RhoGEF (LARG) (11–13). The activation mechanisms of RGS domain-containing RhoGEFs induced by extracellular signals are well known in the case of p115-RhoGEF (14–15). The RGS domain of p115-RhoGEF stimulates the intrinsic GTPase activity of the G<sub>12</sub> or G<sub>13 $\alpha$</sub>  subunit, and activated G<sub>12</sub> or G<sub>13 $\alpha$</sub>  subunit binds to the RGS domain of p115-RhoGEF, thereby enhancing its ability to catalyze guanine nucleotide exchange of RhoA. On the other hand, PDZ-RhoGEF and LARG, but not p115-RhoGEF, both have N-terminal PDZ domains. The PDZ domain is known as a modular domain that binds to specific C-terminal peptide sequences of many membrane proteins (16). PDZ domain-containing proteins function as mediators of clustering of neurotransmitter receptors and ion channels, and then are involved in asymmetric distribution of receptors in epithelial cells (17–20). PDZ-RhoGEF and LARG also bind to the G<sub>12</sub> or G<sub>13 $\alpha$</sub>  subunit via RGS domains in a manner similar to p115-RhoGEF, although the molecular mechanisms controlling the GEF activity are not yet fully understood (8, 21–23). Recently, we and other groups have reported that the PDZ domains of PDZ-RhoGEF and LARG interact directly with the C-terminal domain of Plexin-B1, a Semaphorin-4D (Sema-4D) receptor, and/or the insulin-like growth factor (IGF-1) receptor (24–29). Stimulation of Sema4D or IGF-I-induced RhoA activation through the complex of Plexin-B1 or IGF-I receptors with PDZ-RhoGEF or LARG. These observations suggest that the PDZ domain-mediated interaction between PDZ domain-containing RhoGEFs and Plexin-B1 and/or the IGF-1 receptor may play an important role in the regulation of RhoGEF activity.

In this study, the fact that the C terminus of LPA receptors, LPA<sub>1</sub> and LPA<sub>2</sub>, also have classical PDZ domain interaction motifs prompted us to examine the interaction of LPA<sub>1</sub> and

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<sup>1</sup> The abbreviations used are: LPA, lysophosphatidic acid; Edg, endothelial differentiation gene; HEK, human embryonic kidney; PDZ, PSD-95/Dlg/ZO-1; GST, glutathione S-transferase; MBP, maltose-binding protein; MAP, mitogen-activated protein; GPCR, G protein-coupled receptor; GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; RGS, regulators of G protein signaling.

LPA<sub>2</sub> with the PDZ domains of PDZ-RhoGEF and LARG. We found that the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> directly interacted with the PDZ domains of PDZ domain-containing RhoGEFs, PDZ-RhoGEF, and LARG. Amino acid mutations in the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> abolished LPA-induced Rho activation. Furthermore, LPA-induced RhoA activation was inhibited by overexpression of the PDZ domains of PDZ domain-containing RhoGEFs. These results indicated that PDZ domain-containing RhoGEFs might provide the link between LPA receptors (LPA<sub>1</sub> and LPA<sub>2</sub>) and RhoA activation.

#### EXPERIMENTAL PROCEDURES

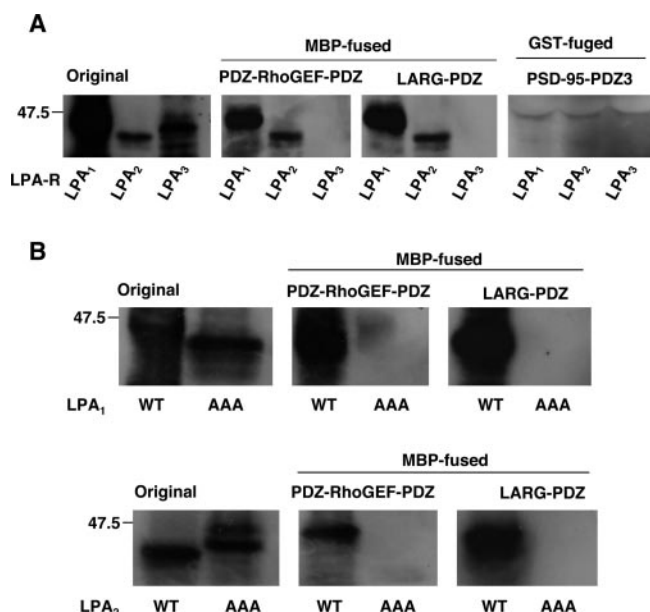
**Construction of Expression Vectors**—Various expression vectors were constructed in pFLAG-CMV (Sigma), pCMV-Myc, pMal-C2 (New England BioLabs), and pGEX5-X1 (Amersham Biosciences) using standard molecular biology methods (30). The cDNAs of LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> were cloned by reverse transcriptase-PCR with mouse kidney cDNA as template using the reverse primer TCTAGACTAAACCACAGAGTGTT, TCTAGATTAAGGGTGGAGTCCATCAG and TCTAGATTAGAGCCGTTTTTATTGCA forward primer GCGGCCG CAATGGCAGCTGCCTCTACT, GCGGCCGCAATGGGCCAGTGTACTACT, and GCGGCCGCAATGAATGAGTGTACTAT. Then the PCR products were subcloned into pFLAG-CMV expression vector. LPA<sub>1</sub> and LPA<sub>2</sub> mutants, pFLAG-CMV-LPA<sub>1</sub>AAA (C terminus three amino acids Ser, Val, Val replaced with three alanines, Ala, Ala, Ala) and pFLAG-CMV-LPA<sub>2</sub>AAA pFLAG-CMV-LPA<sub>1</sub>AAA (C terminus three amino acids Ser, Thr, Leu replaced with three alanines, Ala, Ala, Ala) were prepared with a PCR fragment amplified from the LPA<sub>1</sub> and LPA<sub>2</sub> plasmid using the reverse primers TCTAGACTAAGCCGCGCAGCGTGGTCTGTTGCTGTGAAC and TCTAGACTAAGCCGCGCAGTGTCCATCAGTGGCCGGCC; and forward primers GCGGCCGCAATGGCCAGTGTCTCTACT, and GCGGCCGCAATGGGCCAGTGTACTACT. Then PCR products were subcloned into the pFLAG-CMV expression vector. The cDNAs of human PDZ-RhoGEF and LARG were kindly provided from Kazusa DNA Research Institute (Japan) and Dr. Caligiuri, respectively. pCMV-Myc-RGS-LARG were prepared with a PCR fragment amplified from LARG plasmid using reverse primer (GTCGACGTCCGAAGTCCCTCA) forward primer (ACGCGTGTGCTCCAAATTTTCG). Another PDZ-RhoGEF and LARG various expression vectors were prepared as described (24). pGEX-KG-PSD95-PDZ3 and pCMV-Myc-PSD95-PDZ3\* (this construct contains Src homology 3 (SH3) and guanylate kinase (GK) domains in addition to the third PDZ domain) were generous gifts from Drs. Y. Hata and Y. Takai.

**Chemicals**—Mouse monoclonal antibody to FLAG was purchased from Sigma, and mouse monoclonal antibody to Myc (9E10) was purchased from American Type Culture Collection. For Western blot analysis, primary antibodies were used at a 1:2000 dilution. Immunoreactive proteins were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Laboratories) at a 1:2000 dilution and then visualized using the ECL system (Amersham Biosciences). LPA was purchased from Sigma. Pertussis toxin was purchased from List Biological Laboratories, Inc.

**In Vitro Binding of LPA Receptors to PDZ-RhoGEF and LARG**—HEK293 cells were transfected by the calcium phosphate method with pFLAG-CMV-LPA<sub>1</sub> and LPA<sub>2</sub>, or various mutant plasmids of LPA<sub>1</sub> and LPA<sub>2</sub>. After 48 h, the cells were lysed in 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) Triton X-100), and centrifuged at 100,000 × g for 30 min. 500 μl of the supernatant were incubated with the various MBP-fused proteins fixed on 20 μl of amylose-Sepharose 4B beads (New England BioLabs). After the beads were washed with the lysis buffer, proteins on the beads were subjected to the Western blot analysis.

**Immunoprecipitation**—pCMV-Myc-LARG or pCMV-Myc-LARGΔPDZ was co-transfected with pFLAG-CMV-LPA<sub>1</sub> or -LPA<sub>2</sub>AAA into HEK293 cells by the calcium phosphate method (31). After 48 h of culture, the cells were lysed with the lysis buffer and centrifuged at 100,000 × g for 30 min. The supernatant was incubated with anti-Myc or anti-FLAG antibodies fixed on protein A-Sepharose beads (Amersham Biosciences). After the beads were washed with the lysis buffer, proteins on beads were detected by Western blot with the indicated antibodies.

**Rho Activity Assay**—Various pFLAG-CMV-LPA receptors and/or pCMV-Myc-PDZ-RhoGEF-PDZ- or -LARG-PDZ-transfected HEK293 cells were deprived of serum for 24 h. The cells were treated with or without 10 μM LPA for 2.5 min and then lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). The soluble supernatant



**FIG. 1. Direct interaction of the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> with PDZ domains of PDZ-RhoGEF and LARG.** *A*, lysates from pFLAG-CMV-LPA<sub>1</sub>, -LPA<sub>2</sub>, and -LPA<sub>3</sub>-transfected HEK293 cells were incubated with amylose beads containing MBP-PDZ (PDZ-RhoGEF, LARG) or glutathione beads containing GST-PDZ3 (PSD-95). The beads were washed extensively and eluted with SDS-PAGE sample buffer. Each fraction was subjected to SDS-PAGE (10% polyacrylamide gel) followed by Western blot analysis with anti-FLAG antibody. *B*, lysates from pFLAG-CMV-C terminus mutants of LPA<sub>1</sub> and LPA<sub>2</sub> were used in the affinity assay as described above. WT, wild type; AAA, three amino acids of the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub>; SSV and STL, were substituted by three alanines, AAA.

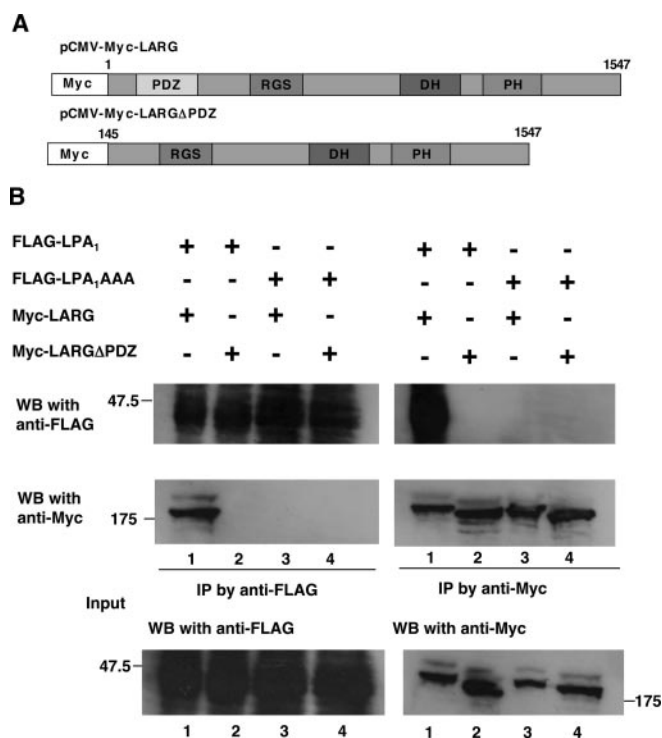
was incubated with GST-rhotekin Rho binding domain (32). The bound RhoA was eluted by boiling in sample buffer for SDS-PAGE and subjected to Western blot analysis with anti-RhoA antibody.

**MAP Kinase Activity Assay**—Various pFLAG-CMV-LPA receptors and/or pCMV-Myc-PDZ-, RhoGEF-PDZ-, or -LARG-PDZ-transfected HEK293 cells were deprived of serum for 24 h. The cells were treated with or without 10 μM LPA for 2.5 min and lysed with buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride). The soluble supernatant was boiled in sample buffer for SDS-PAGE and subjected to Western blot analysis with antiphospho-p44/42 MAPK antibody and p44/42 MAPK antibody (PhosphoPlus p44/42 MAPK antibody kit, New England BioLabs).

#### RESULTS

**LPA Receptors and PDZ Domain-containing RhoGEFs**—LPA signaling is mediated via a family of GPCRs including LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub>. Interestingly, LPA<sub>1</sub> and LPA<sub>2</sub> have a cytoplasmic region with C-terminal amino acid residue motifs of DTL and SSV, respectively, which may be PDZ domain-interacting motifs. PDZ domain-containing RhoGEFs PDZ-RhoGEF, and LARG consist of PDZ, RGS, DH, and PH domains, and their RGS domain may interact with the activated G<sub>12</sub> family causing RhoGEF activation. Because LPA-induced RhoA activation is also known to be mediated by the G<sub>12</sub> family, the possibility was raised that PDZ domain-containing RhoGEFs may interact with LPA<sub>1</sub> or LPA<sub>2</sub> directly and play an important role in LPA-induced RhoA activation.

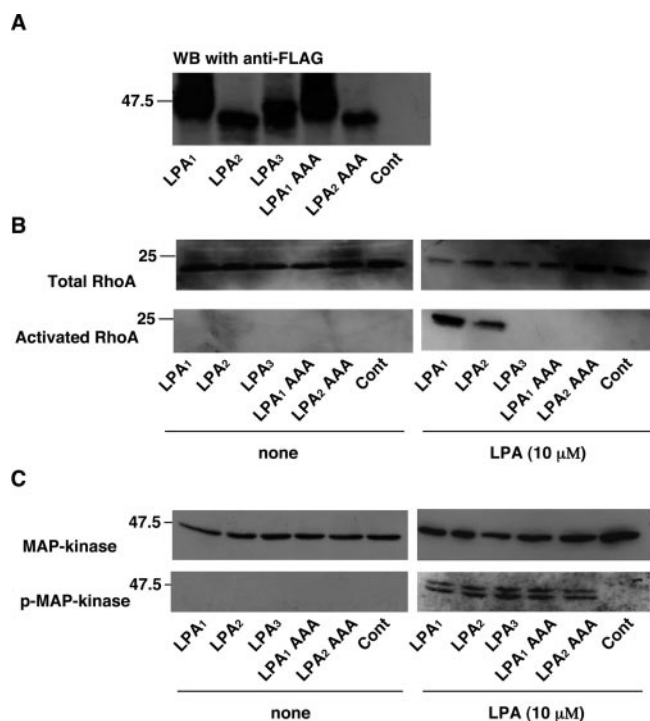
**Interaction of LPA<sub>1</sub> and LPA<sub>2</sub> with the PDZ Domains of PDZ-RhoGEF and LARG**—We first examined the *in vitro* binding of LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> to the PDZ domains of PDZ-RhoGEF and LARG by affinity chromatography. Cell lysates from HEK293 cells transfected with pFLAG-CMV-LPA<sub>1</sub>, -LPA<sub>2</sub>, and -LPA<sub>3</sub> were incubated with MBP-fused proteins of the PDZ domains of PDZ-RhoGEF and LARG. Western blot analysis showed that both LPA<sub>1</sub> and LPA<sub>2</sub> bound MBP-PDZ



**FIG. 2. Co-immunoprecipitation of LPA receptor with PDZ domain-containing RhoGEF.** *A*, schematic representation of the construction of pCMV-Myc-LARG and -LARGΔPDZ, lacking the PDZ domain. *B*, HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub> or -LPA<sub>1</sub>AAA in combination with pCMV-Myc-LARG or -LARGΔPDZ. The cell lysate were incubated with anti-FLAG or anti-Myc antibodies. Protein A-Sepharose bead-associated immunocomplexes were subjected to SDS-PAGE (10% polyacrylamide gel) followed by Western blot analysis with anti-FLAG or anti-Myc antibodies.

domains of PDZ-RhoGEF and LARG but indicated no interaction of LPA<sub>3</sub> with the MBP-PDZ domains (Fig. 1*A*). We examined the specificity of the PDZ domain of PDZ domain-containing RhoGEFs by using the third PDZ domain of PSD-95, which is a scaffolding protein at the postsynaptic density. The third PDZ domain of PSD-95 failed to interact with the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> in addition to LPA<sub>3</sub>. Because LPA<sub>1</sub> and LPA<sub>2</sub> have cytoplasmic regions with C-terminal amino acid residue motifs of DTL and SSV, respectively, we examined the interaction of the C-terminal LPA<sub>1</sub> and LPA<sub>2</sub> mutants with the MBP-PDZ domains of PDZ-RhoGEF and LARG (Fig. 1*B*). Substitution of the three amino acid residues with alanine residues at in the LPA<sub>1</sub> and LPA<sub>2</sub> C-terminal regions completely abolished interactions with the PDZ domains of PDZ-RhoGEF and LARG. These results indicated that LPA<sub>1</sub> and LPA<sub>2</sub> interacted with the PDZ domain of PDZ-RhoGEF and LARG and that the C-terminal motifs of LPA<sub>1</sub> and LPA<sub>2</sub> are essential for binding.

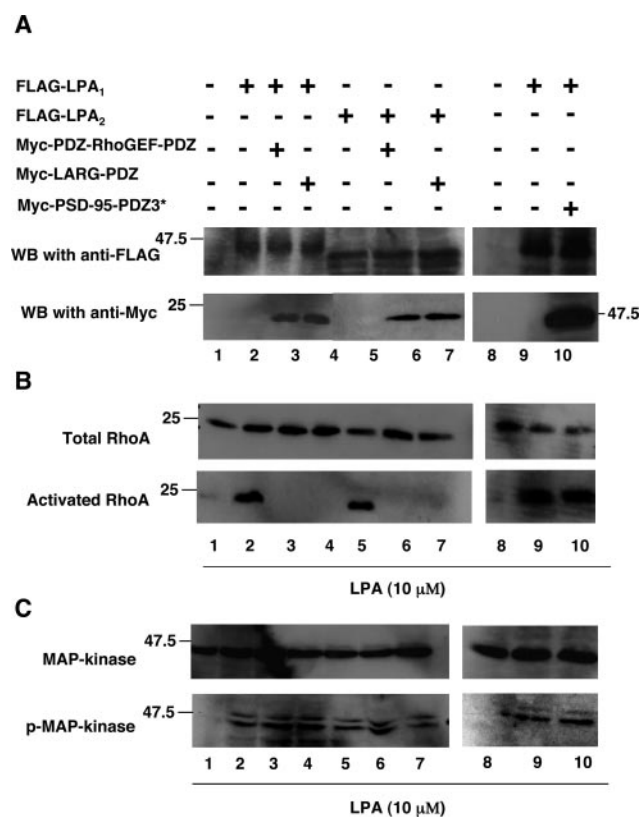
Next, we examined the binding of LPA receptors to PDZ domain-containing RhoGEFs in a cellular environment (Fig. 2). HEK293 cells were co-transfected with pFLAG-CMV-LPA<sub>1</sub> or -LPA<sub>1</sub>AAA and pCMV-Myc-LARG or the mutant lacking a PDZ domain (LARGΔPDZ). The cell lysates were immunoprecipitated with anti-Myc or anti-FLAG antibodies on protein A-Sepharose beads and analyzed by Western blot with anti-Myc or anti-FLAG antibodies. The anti-Myc antibody precipitated both Myc-tagged LARG and LARGΔPDZ from transfected cells, and the Myc-LARG protein co-immunoprecipitated with FLAG-LPA<sub>1</sub> and vice versa. Furthermore, Myc-LARGΔPDZ failed to precipitate FLAG-LPA<sub>1</sub>, and the Myc-LARG protein could not precipitate LPA<sub>1</sub>AAA. Similar results were obtained with LPA<sub>1</sub> and PDZ-RhoGEF, LPA<sub>2</sub> and PDZ-RhoGEF, or LARG (data not shown). These results indicated that the PDZ



**FIG. 3. LPA-induced activation of RhoA and MAP kinase in LPA receptor-transfected cells.** *A*, HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub>, -LPA<sub>2</sub>, -LPA<sub>3</sub>, -LPA<sub>1</sub>AAA, and -LPA<sub>2</sub>AAA, respectively. The expression of each protein was determined by Western blot analysis with anti-FLAG antibody. *B*, these transfected cells were serum-starved for 24 h and then stimulated with or without 10 μM LPA for 2.5 min. The lysates were incubated with GST-rhotekin beads. The bound activated RhoA was subjected to SDS-PAGE (16% polyacrylamide gel) followed by Western blot analysis with anti-RhoA antibody. *C*, these transfected cells were serum-starved for 24 h and then stimulated with or without 10 μM LPA for 2.5 min. The lysates were subjected to SDS-PAGE (10% polyacrylamide gel) followed by Western blot analysis with anti-MAP kinase or antiphospho-MAP kinase antibodies.

domains of PDZ domain-containing RhoGEFs in addition to the C-terminal motifs of LPA<sub>1</sub> and LPA<sub>2</sub> were essential for binding to C terminus of LPA<sub>1</sub> and LPA<sub>2</sub>.

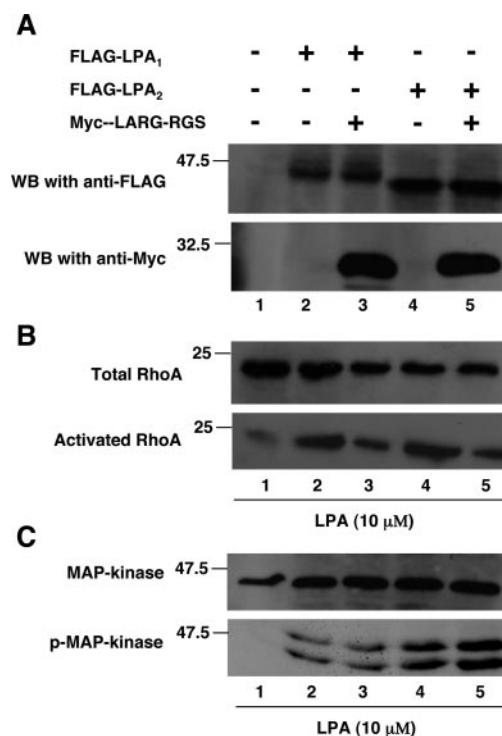
*Interactions of LPA Receptors with PDZ Domain-containing RhoGEFs Are Essential for LPA-induced RhoA Activation but Not for MAP Kinase Activation*—LPA is known as the prototypic GPCR ligand that activates small GTPase, RhoA, and MAP kinase via the G protein (1). We examined whether LPA regulated the RhoA and MAP kinase activities via PDZ domain-containing RhoGEFs. HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub>, -LPA<sub>2</sub>, -LPA<sub>3</sub>, -LPA<sub>1</sub>AAA, and -LPA<sub>2</sub>AAA, respectively (Fig. 3*A*). After LPA stimulation, cell lysates were prepared, and Rho activity was assayed using the GST-Rho binding domain of rhotekin (Fig. 3*B*). LPA-induced RhoA activation was observed in LPA<sub>1</sub>- and LPA<sub>2</sub>-transfected HEK293 cells but not observed in LPA<sub>3</sub>-, LPA<sub>1</sub>AAA-, or LPA<sub>2</sub>AAA-transfected cells. On the other hand, there was no significant activation of RhoA in the absence of LPA in any cells, indicating that overexpression of LPA receptors did not cause constitutive activation of RhoA. Next, we examined the requirement of the interactions between LPA receptors and PDZ domain-containing proteins for LPA-induced MAP kinase activation (Fig. 3*C*). LPA-induced MAP kinase activation was observed in LPA<sub>3</sub>-, LPA<sub>1</sub>AAA-, LPA<sub>2</sub>AAA-transfected cells, as well as in LPA<sub>1</sub>- and LPA<sub>2</sub>-transfected cells. On the other hand, there was no significant activation of MAP kinase in the absence of LPA in any cells indicating that overexpression of LPA receptors did not cause constitutive activation of MAP kinase. These data suggest that of the interaction of the C terminus of



**FIG. 4. Effects of PDZ domains of PDZ-RhoGEF, LARG, or PSD-95 on LPA-induced RhoA and MAP kinase activation.** *A*, HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub>, -LPA<sub>2</sub> in combination with pCMV-Myc-PDZ-RhoGEF-PDZ, -LARG-PDZ, or -PDZ3-PSD-95. The expression of each protein was determined by Western blot analysis with anti-FLAG antibody and anti-Myc antibody. *B*, these transfected cells were serum-starved for 24 h and then stimulated with 10  $\mu$ M LPA for 2.5 min. The lysates were incubated with GST-rhotekin beads. The bound activated RhoA was subjected to SDS-PAGE (16% polyacrylamide gel) followed by Western blot analysis with the anti-RhoA antibody. *C*, these transfected cells were serum-starved for 24 h and then stimulated with 10  $\mu$ M LPA for 2.5 min. The lysates were subjected to SDS-PAGE (10% polyacrylamide gel) followed by Western blot analysis with anti-MAP kinase or antiphospho-MAP kinase antibodies.

LPA receptors with PDZ proteins may play an important role in LPA-induced RhoA activation but LPA-induced MAP kinase activation was independent of the interaction of the C terminus of LPA receptors with PDZ proteins.

Because the expression of both PDZ-RhoGEF and LARG has been confirmed in HEK293 cells (33), we speculated that the exogenous PDZ domains of PDZ-RhoGEF and LARG could compete with endogenous PDZ domain-containing RhoGEFs for binding to the C terminus of LPA receptors in HEK293 cells. We next examined the effect of overexpression of the PDZ domains of PDZ domain-containing RhoGEFs on LPA-induced RhoA activation and MAP kinase activation. HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub> or -LPA<sub>2</sub> with or without the PDZ domains of PDZ-RhoGEF, LARG, and the third PDZ domain of PSD-95 (Fig. 4A). After LPA stimulation, cell lysates were prepared and used in the Rho activity assay and MAP kinase assay. LPA-induced RhoA activation was completely inhibited by overexpression of the PDZ domains of PDZ-RhoGEF and LARG. Whereas overexpression of the third PDZ domain of PSD-95, which did not interact with C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> receptors (Fig. 1A), had no effect on LPA-induced RhoA activation in pFLAG-CMV-LPA<sub>1</sub>- and pFLAG-CMV-LPA<sub>2</sub>-transfected cells (Fig. 4B, data not shown). On the other hand, MAP kinase activation was not altered by overexpression of any PDZ domains (Fig. 4C). It was probable that the



**FIG. 5. Effects of the RGS domain of LARG on LPA-induced RhoA and MAP kinase activation.** *A*, HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub> or -LPA<sub>2</sub> in combination with pCMV-Myc-RGS-LARG. The expression of each protein was determined by Western blot analysis with anti-FLAG antibody and anti-Myc antibody. *B*, these transfected cells were serum-starved for 24 h and then stimulated with 10  $\mu$ M LPA for 2.5 min. The lysates were incubated with GST-rhotekin beads. The bound activated RhoA was subjected to SDS-PAGE (16% polyacrylamide gel) followed by Western blot analysis with the anti-RhoA antibody. *C*, these transfected cells were serum-starved for 24 h and then stimulated with 10  $\mu$ M LPA for 2.5 min. The lysates were subjected to SDS-PAGE (10% polyacrylamide gel) followed by Western blot analysis with anti-MAP kinase or antiphospho-MAP kinase antibodies.

exogenous PDZ domains of PDZ domain-containing RhoGEFs could inhibit interaction of LPA receptors with endogenous PDZ domain-containing RhoGEFs and served as dominant negative mutants for LPA-induced RhoA activation. Taken together, these results suggest that LPA-induced RhoA activation but not MAP kinase activation is mediated by PDZ domain-containing RhoGEFs that associate with LPA receptors.

Finally, we examined the effects of the RGS domain of LARG on LPA-induced RhoA activation to determine whether LPA regulates PDZ domain-containing RhoGEFs via the G<sub>12/13</sub>  $\alpha$  subunit. Because the RGS domain of LARG interacted with the activated  $\alpha$  subunit of G<sub>12/13</sub> (22), we speculated that the exogenous RGS domain of LARG could recruit the activated  $\alpha$  subunit of G<sub>12/13</sub> from endogenous PDZ domain-containing RhoGEFs resulting in weakening the LPA-induced RhoA activation. HEK293 cells were transfected with pFLAG-CMV-LPA<sub>1</sub> or -LPA<sub>2</sub> with or without the RGS domain of LARG (Fig. 5A). After LPA stimulation, cell lysates were prepared and used in the Rho activity assay and MAP kinase assay. Overexpression of the RGS domain of LARG apparently inhibited LPA-induced RhoA activation, though the inhibitory effect was weaker than that of the PDZ domains of LARG (Fig. 5B). On the other hand, LPA-induced MAP kinase activation was not altered by the overexpression of the RGS domain (Fig. 5C). Pertussis toxin, an inhibitor of G $\alpha_i$ , blocked LPA-induced MAP kinase activation but RhoA activation (data not shown). These results supported the idea that PDZ domain-containing RhoGEFs might be involved in LPA-induced RhoA activation and

LPA receptors, LPA<sub>1</sub> and LPA<sub>2</sub>, coupled to G $\alpha_{12/13}$ -linked RhoA activation and G $\alpha_i$ -linked MAP kinase activation.

#### DISCUSSION

**LPA receptors and Their Signaling**—LPA has a wide variety of biological actions, such as cell proliferation, migration, morphological changes, and survival (1). LPA signal transduction is known to mediate via  $\alpha\beta\gamma$  trimeric G protein-coupled receptors including LPA<sub>1</sub>, LPA<sub>2</sub>, and LPA<sub>3</sub> (1–5). LPA receptors couple to three distinct G proteins, G<sub>i</sub>, G<sub>q/11</sub>, and G<sub>12/13</sub> (5–6). The main signaling pathways include: (1) G<sub>i</sub>-mediated stimulation of MAP kinase cascade in cell proliferation; (2) G<sub>q/11</sub>-mediated stimulation of phospholipase C in Ca<sup>2+</sup> mobilization; (3) G<sub>12/13</sub>-mediated activation of RhoA in morphological changes. Recently, it has been identified that the RGS (regulator of G protein signaling) domain-containing RhoGEFs are involved in LPA-induced RhoA activation because RGS domains especially interact with activated  $\alpha$  subunit of G<sub>12/13</sub> (11–13). However, the detailed mechanisms remain unclear.

**PDZ Domain-containing RhoGEFs as LPA Receptor-interacting Molecules**—In this study, we showed that PDZ domain-containing RhoGEFs interacted with LPA receptors, LPA<sub>1</sub> and LPA<sub>2</sub>, through their PDZ domains. The PDZ domains of PDZ domain-containing RhoGEFs seem to be classified as class I, selecting peptides with a hydroxyl amino acid at position –2 (34). The class I PDZ domains interact preferentially with the C-terminal amino acid sequence (S/T)X(V/L) (X represents any amino acid), and PDZ domains bind to the peptides that terminate in a hydrophobic amino acid such as Val, Ile, or Leu. Because the three amino acids of the C terminus of LPA<sub>1</sub> and LPA<sub>2</sub> are SSV and STL, respectively, our finding is consistent with this prediction. Recently, reports have been accumulated that GPCRs interact with PDZ domain-containing proteins. For example,  $\beta_2$ -adrenergic receptor (35), 5-HT<sub>2</sub> serotonin receptor (36), dopamine receptor (37), and LPA<sub>2</sub> receptor (38) have PDZ domain-binding motifs at the C terminus and interact with PDZ domain-containing proteins including the Na<sup>+</sup>/H<sup>+</sup> exchange factor (NHEF), GIPC, PSD-95, etc. These interactions are essential for physiologic signaling, receptor trafficking, and receptor targeting. Thus, the interaction of GPCRs with PDZ domain-containing proteins has recently become the subject of considerable interest.

**Role of PDZ Domain-containing RhoGEFs in the LPA-induced RhoA Activation**—In this study, we showed that LPA did not induce RhoA activation in LPA<sub>1</sub> and LPA<sub>2</sub> mutants, in addition to LPA<sub>3</sub> lacking interaction with PDZ domain-containing RhoGEFs-transfected HEK293 cells. We also showed that overexpression of the PDZ domains of PDZ-RhoGEF and LARG inhibited LPA-induced RhoA activation. These data suggest that the interaction of LPA receptors with PDZ domain-containing RhoGEFs may be necessary for LPA-induced RhoA activation. It has been reported that RGS domain-containing RhoGEFs such as p115RhoGEF, PDZ-RhoGEF, and LARG may play an essential role in cellular RhoA signaling by LPA (9–10). However, it is not clearly understood how these RhoGEFs regulated LPA-induced RhoA activation. The current data indicate that both PDZ-RhoGEF and LARG but not p115RhoGEF are signaling intermediates between the LPA receptor and RhoA activation. Therefore, one model predicts that LPA<sub>1</sub> and LPA<sub>2</sub> can bring associated PDZ domain-containing RhoGEFs into proximity with the appropriate G protein, such as the G<sub>12</sub> family, resulting in RhoA activation. Consequently, it can be assumed that PDZ domain-containing RhoGEFs may be more efficient than p115RhoGEF for LPA-induced RhoA activation.

**The Limited Inhibitory Effects of Overexpression of RGS Domains of LARG**—We have shown that overexpression of the RGS domain of LARG partially inhibited LPA-induced RhoA

activation. Why did RGS domain of LARG have an only insufficient effect on inhibition of LPA-induced RhoA activation in distinction from its PDZ domain? The regulation of subcellular localization of RGS domain-containing proteins is seen to be important for their physiological activities. It has been reported that several RGS domain-containing proteins have additional regulatory motifs, such as PDZ, PX, PTB (phosphotyrosine binding), and GGL (G protein  $\gamma$  subunit-like) domains, and each domain is known to be involved in the regulation of subcellular localization (39). Actually, the interaction of PDZ domains of PDZ-RGS3 with the C terminus of ephrin-B, which is a transmembrane ligand for Eph, was required for its GAP action on CXCR4-mediated G protein signaling (40). Therefore, the limited inhibitory effects of the RGS domain of LARG may be due to the absence of targeting of the RGS domain to the inherent subcellular localization.

Alternatively, these data raise the possibility that the residual activated RhoA may depend on that activated  $\alpha$  subunit of heterotrimeric G protein other than G<sub>12/13</sub> because RGS domains of LARG preferentially interacted with the activated  $\alpha$  subunit of the G<sub>12</sub> family, G<sub>12</sub> and G<sub>13</sub> (12, 22). It has been reported that LPA receptors coupled with G $\alpha_q$  in several cells (4, 41), and activated G $\alpha_q$  also promoted RhoA activation in HEK293 cells (43). Therefore, it is likely that G<sub>q</sub> is also involved in LPA-induced RhoA activation in HEK293 cells. LPA-induced RhoA activation was completely blocked by overexpression of the PDZ domains of PDZ-RhoGEF and LARG, suggesting that PDZ domain-containing RhoGEFs may mediate even the activation of RhoA by G<sub>q</sub>. Recent reports showed that the activated  $\alpha$  subunit of G<sub>q</sub> scarcely interacted with the RGS domains of PDZ-RhoGEF and LARG, but removal of the N-terminal region including the RGS domain enhanced the interaction of G<sub>q</sub> to PDZ domain-containing RhoGEFs (43). These reports suggested that G<sub>q</sub> could activate RhoA through PDZ domain-containing RhoGEFs by a distinct mechanism from G<sub>12</sub> family under LPA stimulation. Anyway, PDZ domain-containing RhoGEFs may play an essential role in LPA-induced RhoA activation in HEK293 cells.

**Determination of Specific Usages of PDZ Domain-containing RhoGEFs**—Recently, it has been reported that thrombin and LPA-induced RhoA activation were mediated by PDZ domain-containing RhoGEFs whereas p115RhoGEF was not involved in either thrombin- or LPA-induced RhoA activation, using the method of oligonucleotide small interfering RNAs (33). These investigators also showed that thrombin and LPA receptors utilized different PDZ domain-containing RhoGEFs, LARG, and PDZ-RhoGEF, respectively. In this study, we showed that the C terminus of LPA receptors, LPA<sub>1</sub> and LPA<sub>2</sub>, could interact with the PDZ domains of both PDZ-RhoGEF and LARG and that overexpression of the PDZ domains of PDZ-RhoGEF and LARG inhibit LPA-induced RhoA activation to the same extent. These results suggest that the PDZ domain mediating the interactions of LPA receptors with PDZ domain-containing RhoGEFs may not determine specificity. How do LPA receptors utilize PDZ-RhoGEF rather than LARG? Whereas PDZ-RhoGEF and LARG are structurally similar to each other, only PDZ-RhoGEF contains a proline-rich motif C-terminally adjacent to the DH/PH domain (21). Consequently, PDZ-RhoGEF differs from LARG in terms of the subcellular localization site on the membrane (21, 24). It is likely that this different subcellular localization may be involved in determination of the coupling receptor selection via the PDZ domain.

Alternatively, it has been reported that the thrombin receptor stimulates G<sub>12</sub> $\alpha$  subunit and that the LPA receptor stimulates G<sub>13</sub> $\alpha$  subunit. The selective coupling of thrombin and LPA receptors to the G<sub>12</sub> family were determined by the N terminus

short sequences of  $G\alpha_{12}$  and  $G\alpha_{13}$  (44). Furthermore, evidence has been provided that tyrosine phosphorylation of PDZ domain-containing RhoGEFs may regulate the activation of RhoA by GPCRs (42, 45). The mechanisms remain unclear but recent reports demonstrate that tyrosine phosphorylation of PDZ domain-containing RhoGEFs does not affect their basal RhoGEF activity, but rather changes their regulation by the  $G_{12}$  family  $\alpha$  subunit (42). The report concludes that  $G_{13}$  stimulates non-phosphorylated LARG, although  $G_{12}$  stimulates the GEF activity of LARG only when LARG was tyrosine-phosphorylated by Tec tyrosine kinase. Thus, these reports suggest that tyrosine phosphorylation of PDZ domain-containing RhoGEFs, in addition to the structural specificity of the  $G_{12}$  family, may determine the selective coupling of thrombin and LPA receptors to the  $G_{12}$  family and PDZ domain-containing RhoGEFs.

Finally, the selective utilization between GPCRs, G proteins, and PDZ domain-containing RhoGEFs remains unclear. Further analysis is necessary for understanding of the mode of selective utilization of them.

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**Physical and Functional Interactions of the Lysophosphatidic Acid Receptors with PDZ Domain-containing Rho Guanine Nucleotide Exchange Factors (RhoGEFs)**

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