Lysophosphatidic acid (LPA) induces actin rearrangement, focal adhesion assembly, and cell migration through the activation of small G protein Rho and its downstream effectors. These diverse cellular responses are mediated by its associated G protein-coupled receptors. However, the mechanisms and specificity by which these LPA receptors mediate LPA actions are still poorly understood. Here we show that LPA stimulation promotes the interaction of the LPA2 receptor with a focal adhesion molecule, TRIP6 (thyroid receptor interacting protein 6)/ZRP1 (zyxin-related protein 1). TRIP6 directly binds to the carboxyl-terminal tail of the LPA2 receptor through its LIM domains. LPA-dependent recruitment of TRIP6 to the plasma membrane promotes its targeting to focal adhesions and co-localization with actin stress fibers. In addition, TRIP6 associates with the components of focal complexes including paxillin, focal adhesion kinase, c-Src, and p130cas in an agonist-dependent manner. Overexpression of TRIP6 augments LPA-induced cell migration; in contrast, suppression of endogenous TRIP6 expression by a TRIP6-specific small interfering RNA reduces it in SKOV3 ovarian cancer cells. Strikingly, the association with TRIP6 is specific to the LPA2 receptor but not LPA1 or LPA3 receptor, indicating a specific role for TRIP6 in regulating LPA2 receptor-mediated signaling. Taken together, our results suggest that TRIP6 functions at a point of convergence between the activated LPA2 receptor and downstream signals involved in cell adhesion and migration.

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The LPA1, LPA2, and LPA3 receptors share high homology in amino acid sequences except for the carboxyl-terminal region, which LPA receptors mediate LPA-induced cell migration are not well understood. Here we show that LPA stimulation promotes the interaction of the LPA2 receptor with a focal adhesion molecule, TRIP6 (thyroid receptor interacting protein 6)/ZRP1 (zyxin-related protein 1). TRIP6 directly binds to the carboxyl-terminal tail of the LPA2 receptor through its LIM domains. LPA-dependent recruitment of TRIP6 to the plasma membrane promotes its targeting to focal adhesions and co-localization with actin stress fibers. In addition, TRIP6 associates with the components of focal complexes including paxillin, focal adhesion kinase, c-Src, and p130cas in an agonist-dependent manner. Overexpression of TRIP6 augments LPA-induced cell migration; in contrast, suppression of endogenous TRIP6 expression by a TRIP6-specific small interfering RNA reduces it in SKOV3 ovarian cancer cells. Strikingly, the association with TRIP6 is specific to the LPA2 receptor but not LPA1 or LPA3 receptor, indicating a specific role for TRIP6 in regulating LPA2 receptor-mediated signaling. Taken together, our results suggest that TRIP6 functions at a point of convergence between the activated LPA2 receptor and downstream signals involved in cell adhesion and migration.

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† The abbreviations used are: LPA, lysophosphatidic acid; FAK, focal adhesion kinase; GFP, green fluorescent protein; siRNA, small interfering RNA; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; EGF, epidermal growth factor; aa, amino acids; LPA-R, LPA1 receptor, and LPA2 receptor.

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Experimental Procedures

Plasmid Construction—The clones containing full-length cDNA sequences of the LPA1 receptor, LPA2 receptor, and TRIP6 were obtained from the I.M.A.G.E. consortium through the American Type Culture Collection (ATCC). One guanine base near the 3′ end of the coding sequence encoding the LPA1 receptor, which was found deleted in the I.M.A.G.E. clone 756526 (21, 22), has been corrected by PCR. The full-length LPA1 receptor cDNA was amplified by reverse transcriptase-PCR using total RNA of SKOV3 ovarian cancer cells as the template. To construct the mammalian expression vectors, different cDNA fragments encoding the LPA1 receptor, a LPA2 receptor mutant lacking the carboxy-terminal tail (aa 296–351), TRIP6 and different truncated TRIP6 mutants were amplified by PCR and inserted into pCMV-Tag2A (Stratagene), pCMV-Tag3A (Stratagene), pEGFP (Clontech), or pHisRed1 (Clontech), respectively, such that these proteins were tagged in-frame with a FLAG epitope, a Myc epitope, a green fluorescence protein (GFP), or a far red fluorescence protein (HisRed1) at their N termini. The complete sequences of each cDNA clone were verified by automatic DNA sequencing. For in vitro binding between TRIP6 and the LPA1 receptor, cDNA fragments encoding TRIP6 or the cytoplasmic tail of the LPA1 receptor (aa 296–351) were inserted in-frame into pGEX-6P-3 and pGEX-6P-1 (Amersham Biosciences), respectively.

To inhibit the expression of endogenous TRIP6, the pSUPER vector was constructed as described (23) and was used to direct the expression of a small interfering RNA (siRNA) of TRIP6 (designated pSUPER-siTRIP6) in mammalian cells, which specifically targets the 19-nt sequence of TRIP6, 5′-GAAAGCTGGTTCACGACATG-3′. Yeast Two-hybrid Screening—For the yeast two-hybrid screening, a cDNA fragment encoding LPA1 receptor-CT was amplified by PCR and inserted in-frame at the 3′-end of Gal4 DNA binding domain of the pAS2–1 yeast shuttle vector (Clontech). The entire cDNA sequences of LPA1-CT were verified by automatic DNA sequencing. This pAS-LPA1-CT was used as the bait to screen a HeLa cell cDNA library (Clontech). After co-transformation of pAS-LPA1-CT and the library plasmids into yeast strain Y190, cells were screened on the plates lacking tryptophan, leucine and histidine supplemented with 25 μg 3-amino-1,2,4-triazole at 30 °C for 3–6 days. Histidine-positive colonies were further screened for positive interaction by β-galactosidase assays. Plasmids harboring cDNA were isolated from positive yeast colonies, transformed into Escherichia coli HB101 by electroporation, and further selected colonies were analyzed by DNA sequencing. The complete sequence of LPA1 receptor-CT was amplified by PCR, inserted into pAS-2, and verified by DNA sequencing. To construct pAS-LPA2-R-CT, an EcoRI/Sall cDNA fragment encoding amino acids 301–634 of the LPA2 receptor was removed from pCMV-FLAG-LPA2-R and inserted into pAS2-1.

Cellular Co-immunoprecipitation and in Vitro GST Pull-down—To examine cellular interaction between TRIP6 and LPA receptors, the vector expressing GFP-tagged or Myc-tagged TRIP6 (wild-type or truncation mutants) was transiently transfected into HEK 293T cells without or with the FLAG-LPA receptor-expressing vector. After starvation in 0.1% fatty acid-free bovine serum albumin Dulbecco’s modified Eagle’s medium overnight, cells were incubated without or with 2 μM LPA for 10 min and harvested in co-immunoprecipitation buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 1 mM EGTA) supplemented with a mixture of protease inhibitors and phosphatase inhibitors. The lysates were briefly sonicated to partially disrupt membrane fractions. Lysates were clarified by centrifugation at 14,000 × g for 10 min. The LPA receptors were immunoprecipitated with anti-FLAG M2 monoclonal antibody-conjugated agarose (Sigma), resolved by SDS-PAGE, and transferred to nitrocellulose membrane for immunoblotting. The co-immunoprecipitated GFP-TRIP6 and Myc-TRIP6 (wild-type or mutants) were detected with a mouse anti-GFP polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Similar experiments were performed to examine co-immunoprecipitation of TRIP6 with paxillin, p130Cas, Myc-Fak, or HA-c-Src in HEK 293T cells. To detect the interaction between endogenous TRIP6 and transfected LPA2 receptors, NIH/3T3 cells were transiently transfected with pCMV-Tag2A or pCMV-Myc-LPA2-R. After transfection or not with 2 μM LPA for 10 min, Myc-LPA2-R from the whole cell lysates was immunoprecipitated with anti-Myc 9E10 monoclonal antibody-conjugated agarose beads (Babco). Co-immunoprecipitated endogenous TRIP6 was detected with a TRIP6-specific polyclonal antibody against amino acids 403–417.

To assess the direct binding of TRIP6 with LPA1-CT or LPA2-CT, GST-LPA1 and GST-TRIP6 were expressed in (BL21) (DE3) (LysS) E. coli and purified by immobilizing the proteins on glutathione-Sepharose 4B beads (Amersham Biosciences). GST-TRIP6 was further digested with PreScission Protease (Amersham Biosciences) to cleave GST. One μg of TRIP6 protein was incubated with 1 μg of GST or GST-LPA1-CT in the binding buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 5 mM DTT, 150 mM NaCl, 0.5% Nonidet P-40) for 3 h at 4 °C. The GST proteins pulled down by GST proteins was resolved by SDS-PAGE and detected with a TRIP6-specific antibody (Transduction Laboratories).

LPA-induced Haptotactic Cell Migration—The haptotactic migration assay was performed in 12-well modified Boyden chambers with 8 μm pore-sized PTFE track-etched membranes (Fisher). SKOV3 cells (American Type Culture Collection) at passages 20–40 were transiently transfected with the expression vector of GFP, GFP-TRIP6, GFP-TRIP6(CT3) (aa 398–476), GFP-TRIP6(CT1) (aa 1–278), or GFP-TRIP6(CT2) (aa 1–350). Cells were trypsinized and washed with Dulbecco’s modified Eagle’s medium containing 1% fatty acid-free BSA to remove residual LPA. Cells at a density of 150,000/well were then placed in the upper chamber of the transwells. The membrane was coated with 10 μg/ml fibronectin overnight. Two μM LPA was added or not in the lower chamber, and cells were allowed to migrate for 6 h. The nonmigrated cells from the top surface were removed with cotton swabs. The filter was then fixed with 3% formaldehyde, cut out and mounted on the glass slide. GFP-positive cells on the whole filter were counted to ensure comparable transfection efficiency among all of the samples. To assess the inhibition of TRIP6 function by siRNA, pEGFP was co-transfected with either pSUPER or pSUPER-siTRIP6 into SKOV3 cells. LPA-induced haptotactic cell migration was assayed as described above. Meanwhile, an aliquot of cells was harvested. The expression of GFP and endogenous TRIP6 in the whole cell lysates was determined by immunoblotting probed with an anti-GFP polyclonal antibody (Santa Cruz Biotechnology) and a TRIP6-specific monoclonal antibody (Transduction Laboratories), respectively.

Immunocytochemistry—To examine LPA-induced co-localization of TRIP6 with the LPA1 receptor, SKOV3 cells were transiently co-transfected with the plasmids expressing HisRed1-TRIP6 and GFP-LPA2-R. Cells were starved in Dulbecco’s modified Eagle’s medium containing 0.1% BSA overnight and then treated with or without 2 μM LPA for 10 min. The images of GFP-LPA2-R and HisRed1-TRIP6 were directly captured by fluorescence microscopy (Axioskop 2; Zeiss). To examine LPA-induced co-localization of TRIP6 with actin or focal adhesion molecules, NIH/3T3 fibroblasts or SKOV3 cells expressing wild-type or mutant GFP-TRIP6 without or with Myc-Fak were plated on coverslips in 6-well plates. After serum starvation overnight, cells were treated with or without 2 μM LPA for 15–20 min and fixed in 3% formaldehyde. After permeabilization with 0.2% Triton X-100, cells were blocked in phosphate-buffered saline containing 2% BSA. To visualize actin cytoskeleton, NIH/3T3 cells were stained with TRITC-phalloidin (Sigma) for 30 min. For immunostaining of vinculin, paxillin, and Myc-Fak, SKOV3 cells were incubated with an anti-vinculin monoclonal antibody (Sigma), anti-paxillin polyclonal antibody (Santa Cruz Biotechnology), and anti-Myc 9E10 monoclonal antibody (Santa Cruz Biotechnology), respectively, for 1 h, and then with the Texas Red-X anti-mouse or anti-rabbit secondary antibody (Molecular Probes, Inc., Eugene, OR) for another 1 h.

Results

The Carboxy-terminal Tail of the LPA1 Receptor, but Not LPA2 or LPA3 Receptors, Interacts with the LIM Domains of TRIP6—Among the amino acid sequences of all three LPA receptors, the carboxyl-terminal region of these receptors is much less homologous. The overall identity of the cytoplasmic tails is 27% between the LPA1 and LPA2 receptors and 17% between the LPA2 and LPA3 receptors. This diversity could account for the receptor specificity in mediating LPA signaling. In an attempt to identify the molecules that are specifically involved in LPA2 receptor-mediated signaling, a fusion protein
containing the carboxyl-terminal tail (aa 296–351) of the LPA2 receptor (designated LPA2-R-CT) and the Gal4 DNA binding domain was used as the bait to screen a HeLa cell cDNA library. A total of 4 million clones were screened, and 28 positive clones encoding the carboxyl-terminal tail of LPA2R (aa 301–364), LPA3R, and LPA2-R (aa 296–351), and LPA2-R (aa 294–353) were inserted into pAS2–1, respectively. pAS or each pAS-LPAR-expressing vector was transformed into yeast Y190 cells with either pGAD or pGAD-TRIP6 encoding TRIP6(ΔN1). The interaction of TRIP6(ΔN1) with LPA2-R-CT was verified by selective growth of transformants on a plate lacking tryptophan, leucine, and histidine supplemented with 3-amino-1,2,4-triazole.

Next, we examined whether TRIP6 directly interacts with the LPA2 receptor in yeast. As shown in Fig. 2, TRIP6 was pulled down by GST-LPA2-R-CT but not GST, indicating a direct interaction between TRIP6 and the carboxyl-terminal tail of the LPA2 receptor.

To examine the association of TRIP6 with different LPA receptors in cells, co-immunoprecipitation of GFP-TRIP6 with the FLAG-tagged LPA1, LPA2, or LPA3 receptor or a LPA2 receptor mutant lacking the cytoplasmic tail (designated LPA2R-CT) and the Gal4 DNA binding domain was used as the bait to screen a HeLa cell cDNA library. A total of 4 million clones were screened, and 28 positive clones encoding the carboxyl-terminal tail of LPA2R (aa 296–476) and LPA3R (aa 351–476) contain LIM domains 1–3 and LIM domains 2 and 3, respectively. The interaction was further verified by selective growth of yeast cells co-expressing both LPA2R-CT and TRIP6(ΔN1) or LPA3R and TRIP6(ΔN1) in a yeast two-hybrid manner (Fig. 3A). The result showed that endogenous TRIP6 specifically binds to the LPA2 receptor but not other LPA receptors.

The yeast two-hybrid screening identified two LPA2R-interacting TRIP6 clones, which encode LIM domains 1–3 and domains 2 and 3, respectively, suggesting that TRIP6 may associate with the LPA2 receptor through its LIM domains. To identify the receptor-binding domain of TRIP6 in cells, a number of vectors expressing different TRIP6 truncation mutants have been generated. Their molecular structures are depicted in Fig. 4E. Indeed, we found that TRIP6(CT2) truncation mutant (aa 339–476), which contains LIM domains 2 and 3, was able to co-immunoprecipitate with the LPA2 receptor (Fig. 3C). Even the TRIP6(CT3) mutant (aa 398–476), containing the LIM3 domain alone, was capable of binding to the LPA2 receptor (Fig. 3C). However, we found that LPA still induced a weak but detectable binding between the LPA2 receptor and a TRIP6(ΔC2) mutant (aa 1–350), which lacks LIM domains 2 and 3 (data not shown). Thus, it appears that all of the LIM domains of TRIP6 contribute to its binding to the LPA2 receptor. Differently from the wild-type TRIP6, these truncated LIM domains interact with the LPA2 receptor in an agonist-independent manner, suggesting that the N-terminal region of TRIP6 regulates this agonist-dependent receptor binding. We further examined the receptor binding ability of a TRIP6(ΔC1) mutant (aa 1–278) lacking the entire LIM domains. As shown in Fig. 3D, LPA stimulation for 10 min promoted co-immunoprecipitation of the LPA2 receptor with wild-type TRIP6 but not the TRIP6(ΔC1) mutant. The LPA2 binding ability of different TRIP6 mutants is summarized in Fig. 4E.
and then treated without or with 2 modified Eagle pCMV-Myc-LPA2R. The LPA2 receptor was immunoprecipitated with bottom panel 40 kDa) and multiple modified forms of LPA receptors (mers (H11011/H11011)). The same blot was then detected with an anti-GFP polyclonal antibody to IB immunoblot (H9004). The LIM domains of TRIP6 bind to the LPA 2 receptor, consistent with previous reports for the role of the LPA2 receptor in LPA-induced morphological changes (24). In addition to the membrane-bound clusters, the complexes of GFP-LPA2R and HcRed1-TRIP6 were also present in the cytosol, suggesting that TRIP6 may be internalized with the activated receptor (Fig. 4A, bottom panel). In the meantime, LPA also promoted TRIP6 targeting to focal adhesions at the leading edge, where, however, the LPA2 receptor was not present (Fig. 4A, bottom panel). Thus, TRIP6 must have dissociated from the LPA2 receptor before targeting to focal adhesions.

To identify the domains of TRIP6 required for LPA-induced targeting of TRIP6 to focal adhesions, we examined co-localization of vinculin with a number of TRIP6 truncation mutants. The molecular structures of these mutants and their abilities to co-immunoprecipitate with the LPA2 receptor are shown in Fig. 4E. It was found that the focal adhesion targeting capacity of TRIP6 was retained when LIMs 1 and -3 domains were deleted (Fig. 4F, TRIP6(ΔC2)), but was almost completely lost when the entire LIM domains were removed (Fig. 4F, TRIP6(ΔC1)). The TRIP6(CT1) mutant, containing all three LIM domains, was able to co-localize with vinculin, whereas the TRIP6(CT2) mutant, containing LIM domains 2 and 3, and the TRIP6(CT3) mutant, containing LIM3 domain alone, only showed a rudimentary capacity to localize to focal adhesions by LPA stimulation (Fig. 4F). All of these results indicate that the LIM1 domain of TRIP6 is the minimal requirement for LPA-induced TRIP6 targeting to focal adhesions; however, other domains appear to cooperate to allow maximal accumulation of TRIP6 at these sites. Since focal adhesion targeting domains of TRIP6 partially overlap with its LPA2R-binding domains, the membr-

Fig. 3. The LIM domains of TRIP6 bind to the LPA2 receptor, but not LPA1R, LPA3R, or a LPA2R mutant lacking its carboxy-terminal tail. A, the GFP-TRIP6 expression vector was transiently transfected into HEK 293T cells alone or with one of the vectors expressing FLAG-tagged LPA receptors (LPA1R, LPA2R, LPA3R, or LPA2RΔC) lacking its C-terminal tail. Cells were starved in Dulbecco's modified Eagle's medium containing 0.1% fatty acid-free BSA overnight and then treated without or with 2 μM LPA for 10 min. The LPA receptors from the whole cell lysates were immunoprecipitated (IP) with anti-FLAG M2 mouse antibody-conjugated agarose beads, and the immunoblot (IB) was probed with an anti-GFP polyclonal antibody to detect co-immunoprecipitated GFP-TRIP6. The same blot was then stripped and reprobed with an anti-FLAG polyclonal antibody to detect immunoprecipitated receptors. Results shown are LPA receptor monomers (~40 kDa) and multiple modified forms of LPA receptors (~50–150 kDa). The bottom panel shows the expression of GFP-TRIP6 in the whole cell lysates. B, LPA2-dependent co-immunoprecipitation of endogenous TRIP6 with transfected Myc-LPA2R in NIH/3T3 cells. NIH/3T3 cells were transiently transfected with either pCMV-Tag3A (mock) or pCMV-Myc-LPA2R. The LPA2 receptor was immunoprecipitated with anti-Myc 9E10 monoclonal antibody-conjugated agarose beads from the unstimulated cells and cells treated with 2 μM LPA for 10 min. The co-immunoprecipitated endogenous TRIP6 was detected with a TRIP6-specific polyclonal antibody. C, co-immunoprecipitation of FLAG-LPA2R with GFP-TRIP6(CT2) (aa 339–476) or GFP-TRIP6(CT3) (aa 398–476), which contain LIM domains 2 and 3 and LIM3 domain, respectively. This experiment was carried out without or with LPA in HEK 293T cells as described above. D, co-immunoprecipitation of FLAG-LPA2R with Myc-TRIP6 or Myc-TRIP6(ΔC1) (aa 1–275) lacking the entire LIM domains. A similar experiment was performed in HEK 293T cells as described above. Myc-tagged TRIP6 and TRIP6(ΔC1) were detected with an anti-Myc polyclonal antibody.
brane-bound TRIP6 might dissociate from the LPA$_2$ receptor through a preferential binding to focal adhesion molecules. It should be noted that not only LPA, but also other ligands such as thrombin, platelet-derived growth factor, and sphingosine 1-phosphate, are able to promote translocation of TRIP6 to focal adhesions (data not shown). Thus, it appears to be a

**Fig. 4.** LPA promotes co-localization of TRIP6 with the LPA$_2$ receptor and TRIP6 targeting to focal adhesions. A, SKOV3 cells were co-transfected with the expression plasmids of GFP-LPA$_2$R and HcRed1-TRIP6. After serum starvation overnight, cells were treated without or with 2 µM LPA for 10 min. The GFP fluorescence of LPA$_2$R and the far red fluorescence of TRIP6 were visualized directly by fluorescence microscopy. B–D, SKOV3 cells transiently expressing GFP-TRIP6 alone or with Myc-FAK were treated without or with 2 µM LPA for 15 min. Cells were fixed, and GFP-TRIP6 was visualized by fluorescence microscopy. The immunostaining of endogenous vinculin (B), Myc-FAK (C), and endogenous paxillin (D) was performed as described under “Experimental Procedures.” E, the molecular structures of different TRIP6 truncation mutants and a summary of their capacity to bind to the LPA$_2$ receptor and co-localize with vinculin. F, SKOV3 cells expressing GFP-TRIP6 mutants were starved overnight and treated with 2 µM LPA for 15 min. The endogenous vinculin was detected with an anti-vinculin antibody, and GFP-TRIP6 mutants were visualized by fluorescence microscopy.
general phenomenon by stimulation with different growth factors. Nonetheless, the interaction of TRIP6 with the LPA₂ receptor seems to provide an additional mechanism for recruiting TRIP6 to the plasma membrane and focal adhesions.

**TRIP6 Co-localizes with Actin by LPA Stimulation** — Next, we examined LPA-induced actin cytoskeletal rearrangement by staining actin with TRITC-phalloidin in NIH/3T3 fibroblasts expressing wild-type or mutant TRIP6. These NIH/3T3 cells express endogenous LPA₁₋₃ receptors by reverse transcriptase-PCR analysis (data not shown). As shown in Fig. 5A, LPA stimulation for 20 min triggered the assembly of actin and co-localization of TRIP6 with actin stress fibers. We further performed deletion analysis to identify the regions of TRIP6 required for actin co-localization. The molecular structures of these mutants and their capacity to co-localize with actin were shown in Fig. 5B. We found that co-localization with actin was retained but was significantly reduced when LIM domains 2 and 3 or the entire LIM domains were deleted (Fig. 5C, TRIP6(ΔC2) and TRIP6(ΔC1)). Among the three LIM domain-containing proteins, TRIP6(CT1), containing LIM domains 1–3, showed the best capacity to co-localize with actin, whereas TRIP6(CT2), containing LIM domains 2 and 3, only remained a weak capacity. The TRIP6(CT3) mutant, containing LIM3 domain alone, almost completely lost its capacity to co-localize with actin (Fig. 5C). Thus, LIM domains 1 and 2 cooperate to allow the most efficient targeting of TRIP6 to actin stress fibers; however, the proline-rich region of TRIP6 also contributes to this capacity.

**TRIP6 Associates with Paxillin, p130 cas, FAK, and c-Src in an Agonist-dependent Manner** — TRIP6 directly binds to the carboxyl-terminal tail of the LPA₂ receptor, raising the possibility that TRIP6 may play an important role in linking LPA₂...
TRIP6 Regulates LPA Signaling

TRIP6 Regulates LPA-induced Cell Migration in SKOV3 Cells—To assess the effect of TRIP6 on LPA-induced haptotactic cell migration, SKOV3 cells were transiently transfected with the expression vector of GFP, GFP-TRIP6, GFP-TRIP6(CT3), GFP-TRIP6(ΔC1), or GFP-TRIP6(ΔC2). The TRIP6(CT3) mutant, containing LIM3 domain alone, was able to bind to the LPA2 receptor (Fig. 3C) but did not localize at focal adhesions (Fig. 4F) or co-localize with actin (Fig. 5C); thus, it might specifically block the function of the LPA2 receptor in cell migration. We found that in the absence of any ligand, the basal number of migrated cells was similar among all of the transfectants. LPA treatment for 6 h induced 3.45-fold increase of cell migration, which was further enhanced to 6.84-fold by TRIP6 but was decreased to 2.11-fold by TRIP6(CT3) (Fig. 7A). The TRIP6(ΔC1) mutant lacks the entire LIM domains for receptor binding (Fig. 3D) and focal adhesion targeting (Fig. 4F). However, it reduced LPA-induced cell migration to 2.41-fold (Fig. 7A), suggesting that the N-terminal proline-rich region of TRIP6 is also important for its function in cell migration. The TRIP6(ΔC2) mutant, which shows a low receptor-binding ability (data not shown) and a weak focal adhesion targeting capacity (Fig. 4F), did not significantly alter LPA-induced cell migration (Fig. 7A). Interestingly, GFP-TRIP6 and GFP-TRIP6(ΔC2), but not GFP, GFP-TRIP6(CT3), or GFP-TRIP6(ΔC1), showed striking punctate patterns in about 5–10% of the migrated cells, suggesting that TRIP6 formed protein complexes with some unidentified signaling molecules (Fig. 7A). It is likely that this complex formation is dependent on integrin, since it could be reproduced by plating TRIP6-expressing cells on fibronectin-coated coverslips (data not shown). The TRIP6(CT3) mutant specifically binds to the LPA2 receptor and partially reduces LPA-induced cell migration, suggesting a role for the LPA2 receptor in mediating LPA-induced cell migration. However, other LPA receptors appear to contribute to this event as well.

Previously, it has been shown that displacing zyxin from its normal subcellular location perturbs cell migration (29). In addition, overexpression of LPP increases EGF-induced cell migration in vascular smooth muscle cells (30). These observations suggest an intrinsic role for these zyxin family members in cell migration. To investigate whether TRIP6 has similar effects on cell migration by stimulation with other ligands, thrombin-induced cell migration was carried out in SKOV3 cells overexpressing either GFP or GFP-TRIP6. We found that thrombin-induced haptotactic cell migration was not significantly altered by overexpression of TRIP6 in SKOV3 cells (Fig. 7B). Other ligands, such as platelet-derived growth factor and sphingosine 1-phosphate, mildly induced haptotactic cell migration in SKOV3 cells, which was not affected by TRIP6 either (data not shown). These results suggest that although TRIP6 can be targeted to focal adhesions by different growth factors and is probably one of the components directly involved in cell migration, TRIP6 specifically augments LPA-induced cell migration presumably through LPA-promoted interaction with the LPA2 receptor.

To verify the physiological function of TRIP6 in LPA-induced cell migration, we further knocked down the expression of
endogenous TRIP6 by a TRIP6-specific siRNA. In this experiment, the pEGFP expression vector was transiently transfected into SKOV3 cells with either the pSUPER empty vector (23) or pSUPER-siTRIP6 expressing TRIP6 siRNA. A haptotactic cell migration assay was performed as described above, and GFP-positive cells migrated to the bottom side of the filter were counted by fluorescence microscopy. Our result showed that suppression of endogenous TRIP6 expression by TRIP6-specific siRNA significantly attenuated LPA-induced cell migration from 4.49- to 1.67-fold (Fig. 7C). The reduction of endogenous TRIP6 expression in cells transfected with pSUPER-siTRIP6 (lane 2) versus pSUPER (lane 1) was 50% (Fig. 7C). It should be noted that the extent of TRIP6 siRNA-mediated knockdown of endogenous TRIP6 would be much greater, since the best transfection efficiency of SKOV3 cells we could reach was ~50%. Taken together, these results suggest a pivotal role
TRIP6 Regulates LPA Signaling

TRIP6 in linking LPA2 receptors and the downstream signals required for cell adhesion and migration.

**DISCUSSION**

To gain insights into the understanding of the mechanisms by which the LPA2 receptor mediates LPA signaling, we have identified TRIP6 as a novel LPA2 receptor-interacting protein and showed that TRIP6 regulates LPA-induced cell migration in SKOV3 ovarian cancer cells. TRIP6 is a newly identified zyxin family member, which has been postulated to be involved in cell migration (12). However, so far its function has not yet been fully understood. In this paper, we demonstrated that TRIP6 associated with the membrane-bound and internalized LPA2 receptor by LPA stimulation but was dissociated from the LPA2 receptor when it was translocated to focal adhesions. Moreover, LPA induced co-localization of TRIP6 with actin and promoted its association with the major signaling components known to regulate cell adhesion and migration, including c-Src, FAK, p130Cas, and paxillin. Using deletion analysis, we showed that LIM1 domain was the minimal requirement for its focal adhesion targeting, whereas both LIM domains 1 and 2 and the N-terminal proline-rich region contributed to its co-localization with actin.

In this report, we provided evidence for the direct involvement of TRIP6 in cell migration by showing that suppression of endogenous TRIP6 expression by a TRIP6-specific siRNA attenuated LPA-induced cell migration. Cell migration is a dynamic process that requires a tight coordination between cell attachment and detachment from extracellular matrix. Thus, a moderate strength of cell adhesion would be the most favorable for cell migration (31). This process requires an appropriate regulation of the assembly and disassembly of focal complexes. Therefore, overexpression of one focal adhesion component may alter cell migration depending on the formation and turnover of focal complexes. It may also be dependent on different cellular contexts. This might explain why previously it has been shown that zyxin and TRIP6 interacted with p130Cas and Cbl/HIF1; however, overexpression of TRIP6 in 10T1/2 cells slowed cell migration, perhaps by preventing the interaction of p130Cas and the downstream signaling molecule such as Crk (12). In contrast, recently LPP has been shown to promote EGF-induced cell migration in vascular smooth muscles (30). Different from these reports, here we demonstrated that overexpression of TRIP6 enhanced LPA-induced, but not thrombin-induced, cell migration in SKOV3 ovarian cancer cells. It should be noted that our results do not exclude the involvement of TRIP6 in other growth factor-mediated cell migration. Instead, our results suggest that TRIP6 plays a role in actin dynamics and is a component of focal complexes involved in cell adhesion and migration. Nonetheless, it seems plausible that such receptor binding of TRIP6 promotes its recruitment to the plasma membrane and positions it in proximity to other signaling molecules, thereby enhancing LPA-promoted cell migration. On the other hand, TRIP6 may serve as an adaptor by recruiting unidentified proteins to the receptor-occupied protein complexes and activate LPA-induced downstream signaling. Since TRIP6 only interacts with the LPA2 receptor and not other LPA receptors, it is of further interest that different LPA receptors may mediate LPA signaling through different mechanisms. For example, it has recently been shown that the LPA2 receptor couples to a G1-phosphoinositide 3-kinase-Tiam1 pathway to activate Rac, and thereby stimulates cell motility (32). Another report demonstrated that the LPA3 receptor is required for LPA-induced, laminin-mediated cell migration in some ovarian cancer cells (33).

The LIM domains of TRIP6 display a high degree of sequence homology with LPP and zyxin, which also contain three LIM domains at their carboxyl terminus (13). The overall identity of all three LIM domains is 71.9% between TRIP6 and LPP and 61.5% between TRIP6 and zyxin. Although all three proteins possess the proline-rich sequences at their N terminus, the identity of this region is much less homologous. Given the high degree of sequence homology in their LIM domains, LPP and zyxin may also bind to the LPA2 receptor. Indeed, we found that both LPP and zyxin were able to co-immunoprecipitate with the LPA2 receptor, but not the LPA3 or LPA4 receptor, in an agonist-dependent manner. Whether LPP and zyxin also play a role in LPA2 receptor-mediated signaling remains to be elucidated.

The function of LPA in cell migration plays a central role not only in wound healing and embryonic development but also in the progression of tumors from a noninvasive to an invasive and metastatic phenotype (1). In particular, the levels of LPA and the expression of LPA2 and LPA3 receptors are elevated in ovarian cancer cells but not in normal ovarian epithelial cells (28, 34). These observations implicate a potential role for LPA signaling in ovarian cancer progression. The present studies provide evidence for a physiological role of TRIP6 in regulating LPA-induced cell migration in SKOV3 ovarian cancer cells. Furthermore, TRIP6 may internalize with the activated LPA2 receptor and shuttle between focal adhesions, cytoplasm, and nucleus, such that it may relay LPA2 receptor-mediated signals from the plasma membrane to nucleus and regulate LPA-dependent gene expression. The interaction between TRIP6 and the LPA2 receptor but not other LPA receptors might help in understanding the specific role of the LPA2 receptor in ovarian tumor progression in the future.

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