ROLE OF TRIP6 IN LPA-INDUCED CELL MIGRATION

by

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ABSTRACT

The LIM domain-containing Thyroid Receptor-Interacting Protein 6 (TRIP6) is a zyxin family member that has been implicated in actin dynamics and cell motility. In this study, we have demonstrated that LPA stimulation promotes the recruitment of TRIP6 to the activated LPA2 receptor, and induces the association of TRIP6 with the components of focal complexes, including paxillin, p130Cas, FAK and c-Src. Overexpression of TRIP6 enhances LPA-induced SKOV-3 ovarian cancer cell migration; in contrast, suppression of the endogenous TRIP6 expression by its specific small interfering RNA inhibits it, suggesting a physiological role for TRIP6 in LPA-induced cell migration. The function of TRIP6 in cell motility is regulated by c-Src-mediated phosphorylation at Tyr-55. This phosphorylation is required for TRIP6 coupling to Crk SH2 domain and ERK activation, thereby enhancing LPA-induced morphological changes and chemotaxis. We further demonstrate that TRIP6 phosphorylation and function are negatively modulated by a PTP-L1/FAP-1 (Fas-associated phosphatase-1)-dependent mechanism. PTP-L1 dephosphorylates phosphotyrosine-55 residue of TRIP6 in vitro, and inhibits LPA-induced tyrosine phosphorylation of TRIP6 in cells. In contrast, deletion of the carboxyl-terminal LIM3 and PDZ-binding domains of TRIP6 disrupts the interaction with PTP-L1, and abolishes PTP-L1-mediated negative regulation of TRIP6 phosphorylation. As a result, LPA-induced association of TRIP6 with Crk, and the function of TRIP6 in promoting LPA-induced morphological changes and cell migration are inhibited by PTP-
L1. Taken together, a switch from c-Src-mediated phosphorylation to PTP-L1-dependent dephosphorylation serves as a negative regulatory mechanism to control TRIP6 function in LPA-induced cell migration.
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<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine 5’-monophosphate kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>Asep</td>
<td>APC-stimulated exchange factor</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 5’-monophosphate</td>
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<tr>
<td>CasL/HEF1</td>
<td>Cas Large/ Human Enhancer of Filamentation 1</td>
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<td>Cdc42</td>
<td>Cell division cycle 42</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CFP</td>
<td>Cyano fluorescent protein</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRIP2</td>
<td>Cysteine-rich intestinal protein 2</td>
</tr>
<tr>
<td>Crk</td>
<td>Chicken tumor virus 10 regulator of kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dock180</td>
<td>Dedicator of cytokinesis 180</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EDG</td>
<td>Endothelial differentiation gene</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAP-1</td>
<td>Fas-associated phosphatase 1</td>
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<tr>
<td>FERM domain</td>
<td>an acronym of “band 4.1, ezrin, radixin, and moesin</td>
</tr>
<tr>
<td>Gal4</td>
<td>β-Galactoside α-2,3-sialyltransferase 4</td>
</tr>
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<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GPR23</td>
<td>G protein-coupled receptor 23</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine 5’-triphosphate hydrolase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEK 293T</td>
<td>Human epithelial kidney 293 cells containing SV40 large T antigen</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblot</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KIND</td>
<td>Kinase noncatalytic C lobe domain</td>
</tr>
<tr>
<td>LIM</td>
<td>Lin-11, Isl-1, and Mec-3</td>
</tr>
<tr>
<td>LIMD1</td>
<td>LIM domain containing 1</td>
</tr>
<tr>
<td>LMW-PTP</td>
<td>Low molecular weight PTP</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPP</td>
<td>Lipoma preferred partner</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEK1</td>
<td>MAPK/ERK kinase 1</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light-chain kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Message RNA</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>Nod1</td>
<td>Nucleotide-binding oligomerization domain containing 1</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>OpaP</td>
<td>Opacity-associated protein</td>
</tr>
<tr>
<td>p130&lt;sup&gt;cas&lt;/sup&gt;</td>
<td>p130 Crk-associated substrate</td>
</tr>
<tr>
<td>PARG-1</td>
<td>PTPL1-associated Rho GTPase activating protein 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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</table>
PDZ an acronym of post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg), and zo-1

PI3K Phosphoinositide 3-kinase
PKC Protein kinase C
PLC Phospholipase C
PLD Phospholipase D
PPARγ Proliferator-activated receptor-γ
PRK2 Protein kinase C-related kinase-2
PTP Protein tyrosine phosphatase
RhoGEF Rho-specific guanine nucleotide-exchange factor
RIL Reversion-induced LIM
RIP2 Receptor-interacting protein 2
RNA Ribonucleic acid
RXR Retinoid X receptor
S1P Sphingosine-1-phosphate
S.E. Standard errors
SH2 Src homology 2
siRNA small interfering RNA
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SrfH/SseI SsrB regulated factor H/Salmonella secreted effector I
SYF cells Cells lacking c-Src, Fyn, and Yes of the Src family kinases
TGF-β Transforming growth factor β
Tiam1 T-lymphoma invasion and metastasis 1
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>TRβ1</td>
<td>Thyroid hormone receptor β1</td>
</tr>
<tr>
<td>TRIP6</td>
<td>Thyroid hormone receptor interacting protein 6</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>ZRP1</td>
<td>zyxin-related protein 1</td>
</tr>
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CHAPTER 1

INTRODUCTION

Introduction of LPA and LPA signaling

Lysophosphatidic acid (LPA) is a lysophospholipid, which mediates diverse physiological and pathophysiological activities including mitogenesis, cell cycle progression, differentiation, cell survival, platelet aggregation, inflammation, angiogenesis, wound healing, cell migration, and tumor cell invasion [1, 2]. LPA is generated naturally as an albumin-bound serum factor during blood clotting from activated platelets, and is produced normally by fibroblasts, mesothelial cells, and adipocytes [3, 4]. It is abundant in serum but not in plasma, and the biological concentration of LPA in serum is ~1-10 µM [5]. In addition to serum, it also accumulates in other biological fluids such as aged plasma and ascites induced by ovary tumors [6]. LPA is a growth factor-like bioactive phospholipid and needs membrane-bound G protein coupled receptors to transduce its signals. Since 1996, five membrane-bound LPA receptors have been identified. The first three identified LPA receptors (LPA₁, LPA₂ and LPA₃) belong to the endothelial differentiation gene (EDG) family [2], while the other two LPA receptors, LPA₄/GPR23 and LPA₅/GPR92, are structurally distinct from the EDG family members [7, 8]. In 2003, a nuclear receptor, proliferator-activated receptor-γ (PPARγ), was also identified as an intracellular target of LPA [9], indicating the GPCR-independent signaling of LPA.
The tissue expression patterns of LPA1, LPA2 and LPA3 receptors are summarized in Table 1 [2, 10]. Unlike the universal expression of LPA1, LPA2 and LPA3 receptors, the mRNA levels of LPA4 and LPA5 are less abundant. Overall, LPA4 is expressed in ovary and LPA5 is detected in small intestine, spleen, dorsal root ganglion, and embryonic stem cells [7, 8].

Table 1

Expression patterns of LPA receptors

<table>
<thead>
<tr>
<th>LPA receptors</th>
<th>Expression</th>
<th>Little or no expression</th>
</tr>
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<tr>
<td>LPA1-mouse human</td>
<td>ventricular zone of embryonic cerebral cortex, oligodendrocytes, Schwann cells, testes, lung, heart, intestine, spleen, kidney, thymus, stomach colon, placenta, prostate, ovary, pancreas, skeletal muscle</td>
<td>Liver lung, thymus, peripheral blood leukocytes, liver</td>
</tr>
<tr>
<td>LPA2-mouse human</td>
<td>testes, kidney, embryonic brain, heart, lung, spleen, thymus, stomach, adult brain testes and peripheral blood leukocytes</td>
<td>liver, small intestine, and skeletal muscle heart, brain, placenta, lung, liver, skeletal muscle, kidney, ovary, small intestine, colon</td>
</tr>
<tr>
<td>LPA3-mouse human</td>
<td>testes, kidney, lung prostate, testes, pancreas, heart, lung, ovary</td>
<td>heart, small intestine, stomach, spleen, perinatal or adult brain, brain, small intestine, colon, liver, placenta, skeletal muscle, kidney, spleen, thymus, peripheral blood leukocytes</td>
</tr>
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</table>
**LPA Metabolism**

Several LPA subtypes are detected in activated platelets, including saturated (stearoyl (18:0), palmitoyl (16:0)) and unsaturated (oleoyl (18:1), linoleoyl (18:2), arachidonoyl (20:4)) acyl-chains [4, 11]. LPA is generated by enzyme catalysis through different pathways (Fig 1.1) [12-14]. The first one is deacylation of phosphatidic acid by phospholipase A1 and A2 (PLA₁ and PLA₂). The second one is cleavage of lysophospholipid by lyso-phospholipase D (lyso-PLD), and the third one is mild oxidation of low-density lipoproteins [12-14]. The phosphatidic acid-selective PLA₁ and secretory type-II PLA₂ cleave primarily the surface-exposed phosphatidic acids of microvesicles, for example, from activated inflammatory cells. However, the main source of extracellular LPA is cleaved by lyso-PLD from lysophospholipid, such as lysophosphatidylcholine (LPC). Recently, it has been identified that the cell motility-stimulating factor, autotoxin, is the lyso-PLD responsible for generating LPA in plasma and serum [15, 16]. The plasma levels of LPA in autotoxin<sup>−/−</sup> heterozygous mice were only half compared to that in wild type mice [17, 18], but the levels of S1P (sphingosine-1-phosphate, another lysophospholipid) were not affected [19]. Autotoxin-deficient mice are embryonic lethal with major vascular defects, allantois malfunction, neural tube defects, and asymmetric head-folds [17, 18]. These phenotypes are much more severe than LPA receptor knockout mice (see below), which may be due to the general effect of losing LPA during early embryogenesis [17]. In addition to the pathways described above, an acylglycerol kinase was recently reported to phosphorylate monoacylglycerol and diacylglycerol to form LPA and phosphatidic acid, respectively. This enzyme was primarily located in mitochondria, and its overexpression increased LPA production and secretion [20].
The Signaling Pathways of LPA Receptors

The LPA₁, LPA₂ and LPA₃ share 50-54% amino acid identity. They couple to different G proteins to mediate diverse LPA signals [2, 12]. In general, all three LPA receptors can couple to Gᵢ and Gₒ, but only LPA₁ and LPA₂ can associate with G₁₂/₁₃ to mediate small GTPase Rho activation, which results in cytoskeletal rearrangement and cell migration. Through coupling to Gᵢ protein, LPA activates the Ras-MAPK and PI3K-AKT pathways to promote cell cycle progression and cell survival. It also reduces cyclic AMP (cAMP) production by inhibition of adenylyl cyclase (AC) activity. The LPA receptors couple to Gₒ to mediate phospholipase C (PLC) activation, which in turn, induce calcium mobilization and protein kinase C (PKC) activation. The function of each LPA receptor of the EDG family has been studied by heterologous expression approaches in B103 (rat neuroblastoma) and RH7777 (rat hepatoma) cell lines, where the transcripts of these LPA receptors are undetectable [21]. The biological functions of LPA₁ and LPA₂ receptors have been demonstrated by targeted-disruption of these genes in mice. The LPA₁-null mice show ~50% perinatal lethality. Although the other 50% LPA₁-null mice are fertile, they show reduced body mass, head/facial malformation, and increased death of Schwann cells [22]. The exencephaly and frontal brain hemorrhage may contribute to a small proportion of embryonic death, and the suckling problems due to olfactory defects partly account for postnatal lethality. The cellular signaling events that have been affected in LPA₁ knockout mice include reduced PLC activation and Ca²⁺ mobilization, and abolished AC inhibition in mouse embryonic fibroblasts (MEFs) following LPA treatment. Impaired cluster compaction and reduced cell proliferation in response to LPA
were also observed in dissociated embryonic LPA1−/− neuroblasts [22]. The phenotypes of LPA2 knockout mice, on the other hand, are not as severe as LPA1 knockouts [23]. They are viable and fertile, but show reduced PLC activation and Ca2+ mobilization in MEFs after LPA stimulation. The phenotypes of LPA1−/− and LPA2−/− double knockout mice are similar to that of LPA1-null mice, but with a higher incidence of frontal hematoma. In addition to PLC activation and Ca2+ mobilization, other cellular signaling impacts in response of LPA in MEFs include severely reduced stress fiber formation, and elimination of JNK and Akt activation [23, 24].

Recently, two structurally different GPCRs have also been identified as LPA receptors, including LPA4/P2y9/GPR23 [7] and LPA5/GPR92 [8]. These two receptors share ~35% amino acid identity with each other, while only 20-24% with LPA1, LPA2 and LPA3. With LPA4 expressed in RH7777 cells, it is functionally distinct from LPA1, LPA2 and LPA3. The cAMP and [Ca2+]i (intracellular Ca2+ concentration) were elevated, which can be mediated through coupling to Gs and Gq protein, respectively. Expression of the LPA5/GPR92 in RH7777 cells stimulates stress fiber formation, and in B193 cells increases neurite retraction via G12, G13 and Rho kinase activation. Moreover, LPA4 can mediate the elevation of [Ca2+]i via Gq and increase cAMP levels via Gi. In conclusion, LPA4 and LPA5 receptors mediate LPA signaling by coupling to Gs protein, which is different from other LPA receptors of the EDG family.

In addition to these G protein coupled LPA receptors, LPA has been found to bind to an intracellular receptor, PPARγ [9]. PPARγ is a transcriptional regulator, which controls gene expression involved in glucose and fatty acid metabolism, adipocyte differentiation, and vasculature inflammation. The antidiabetic thiazolidinediones, anionic fatty
acids and their oxidized derivatives are endogenous ligands that activate PPARγ [25]. LPA, but not phosphatidic acid, can compete with thiazolidinedione and rosiglitazone for PPARγ binding and stimulate PPARγ-responsive gene expression. LPA by itself may be able to enter the cells directly and associate with the intracellular target, PPARγ. In preadipocytes that express high levels of PPARγ, interestingly, LPA does not increase the expression of two PPARγ-sensitive genes. Instead, it decreases PPARγ2 expression and inhibits the rosiglitazone-mediated activation of gene transcription and reduces triglyceride accumulation. These anti-adipogenic effects are not observed in LPA1 knockout cells, suggesting that LPA1 may be involved in down-regulation of PPARγ2. These opposite LPA effects in preadipocytes may be due to the high expression and activity of ecto-lipid phosphate phosphatases in these cells, which prevent the entry of LPA to the nucleus to activate PPARγ.

LPA and Cell Migration

One of the most important functions of LPA is to regulate cell motility. It has been known that LPA receptors regulate cytoskeletal organization and cell shape via coupling to G12/13 protein and the activation of small Rho GTPases, including RhoA, Cdc42 and Rac [26-28]. The activation of RhoA promotes actomyosin-driven contractility, Cdc42 mediates filopodia protrusion, and Rac induces lamellipodium ruffling and drives cell movement. An efficient cell migration requires the coordination of all these factors. LPA activates RhoA by direct binding of three RhoGEFs (Rho-specific guanine nucleotide-exchange factors) to G12/13, and promote Rho-GTP formation. The GTP-bound
RhoA in turn activates Rho kinase, which results in actomyosin-based contractile, such as cell rounding, neurite retraction, and tight junction opening.

LPA has been demonstrated to promote cell migration in some cell types including endothelial cells and fibroblasts (for example, β1AGD25 fibroblastic cells that derived from embryonic stem cells, M17 fibroblastic cells, and human skin fibroblasts, etc.). It also stimulates cell migration in some cancer cells, such as T-24 bladder carcinoma cells, breast carcinoma cells, human transitional-cell carcinoma cells, and rat hepatoma cells, etc (for reviews, see [29] and [13]. Some other cell types, such as vascular smooth muscle cells and most of the endothelial cells, however, do not migrate by LPA stimulation [30]. It suggests that LPA may have both chemokinetic and chemostatic abilities depending on the diverse signaling pathways in different cell types [29].

In addition to the activation of Rho family, LPA has been shown to activate several other molecules that are important in the regulation of cell migration. For example, in Swiss 3T3 cells, breast carcinoma cells and hepatoma cells, LPA induces tyrosine phosphorylation of several focal adhesion molecules, such as paxillin, p130Cas (crk-associated substrate) and focal adhesion kinase (FAK) [31], which are important for the actin filament network organization. Moreover, LPA induces gene expression of matrix metalloproteinases (MMP) [32], which play a critical role in cell migration and cancer cell invasion. Another downstream effector of LPA known to regulate cell motility regulation is ERK [33]. LPA activates the MAPK-ERK pathway via coupling to G_{i/o}-protein and stimulates the activation of the small GTPase, Ras. Ras activates Raf, which phosphorylates ERK, and activates it. LPA-induced ERK activation has been shown to be involved in human pancreatic carcinoma cell migration [33-35]. Phosphorylated (activated) ERK is tran-
siently translocated to the newly forming focal contacts at the leading edge of the migrating cells by LPA-stimulation. Inhibition of ERK activation and its translocation to the focal contacts impaired LPA-induced tumor cell migration and actin reorganization. All these results show the importance of LPA signaling in cell motility.

Although LPA receptors have been identified since 1990, the specificity and mechanisms of how different LPA receptors mediate these diverse cellular signaling events are poorly understood. It has been known that LPA2 is up-regulated in ovarian tumor cells [36, 37], and an LPA2-frame shift mutation, which generates an extra 31 amino acids in the carboxyl-terminus, has been identified in ovarian cancer [38]. This implicates a specific role of the LPA2 receptor in ovarian tumor progression. Although the LPA1, LPA2 and LPA3 receptors show high amino acid identity, the carboxyl-terminal tails of these receptors are structurally distinct from each other, suggesting that this region may specifically regulate the unique protein-protein interactions and functions of each receptor. To determine the differential regulation of LPA signaling by different LPA receptors, we used the C-terminal tail of LPA2 as bait to perform the yeast-two hybrid screening, and identified TRIP6 as a LPA2-specific interacting molecule. In this study, we demonstrate that TRIP6 regulates LPA-induced cell migration by reversible tyrosine phosphorylation and dephosphorylation of TRIP6, which is regulated by c-Src tyrosine kinase and PTPL1 phosphatase.

Introduction of TRIP6

TRIP6 (thyroid hormone receptor interacting protein 6) was first identified as a thyroid hormone receptor-interacting protein in a yeast-two hybrid screen using the com-
plex ligand binding/dimerization/ transcriptional activation domain of the rat thyroid hormone receptor TRβ1 as the bait [39]. In addition to the thyroid hormone receptor, TRIP6 also interacts with retinoid X receptors (RXRs) in a ligand dependent manner. Later, TRIP6 was also identified as an interacting protein of the tyrosine phosphatase PTP-1E/PTPL1/PTP-BL [40], the opacity-associated protein (OpaP) of Neisseria gonorrhoeae [41], the adaptor protein RIL [42], the v-Rel oncoprotein [43], p65 subunit protein of NF-κB, receptor-interacting protein 2 (RIP2) [44], glucocorticoid receptor [45], AMP kinase (AMPK) [46], and endoglin [47], a component of the transforming growth factor β (TGF-β) receptor complex. These observations suggest that TRIP6 may be involved in diverse cellular functions through the interaction with a variety of TRIP6 partners.

TRIP6 is also known as zyxin-related protein 1 (ZRP1), a member of the zyxin family [40, 48]. Other members of this family include zyxin, LPP (lipoma preferred partner) [49], Ajuba [50] and LIMD1 (LIM domain containing 1) [51]. All members of the zyxin family contain a proline-rich domain and the nuclear export signal (NES) at the N-terminal region, and three LIM (named after Lin-11, Isl-1, and Mec-3) domains at the C-terminus [48, 52]. The LIM domain consists of two cysteine-rich zinc fingers with the conserved sequences $\text{CX}_2\text{CX}_{16-23}\text{HX}_2\text{CX}_2\text{CX}_2\text{CX}_16\text{C/H/D}$ [48]. The LIM domains are important in mediating protein-protein interactions. It has been demonstrated that these LIM domain-containing proteins play different roles in intracellular signaling, transcriptional regulation, and cellular differentiation during development [48]. The zyxin family proteins and other LIM domain-containing proteins such as the paxillin family are focal adhesion molecules, which build up multiple interactions and signaling networks in regulating cell adhesion, spreading and migration. These focal adhesion pro-
teins may also play an important role in transducing signals from extracellular matrix into the nucleus to regulate gene transcription, cell proliferation, differentiation and apoptosis [53].

The human TRIP6 gene is mapped to chromosome 7q22 [48], which is commonly deleted in malignant myeloid neoplasm and uterine leiomyoma. The relationship of TRIP6 and these tumors needs to be further explored. TRIP6 mRNA and protein are highly expressed in kidney, heart, liver, lung, fat tissue ovary and pancreas, and with weak expression or absent in placenta, brain, and skeletal muscle ([40, 48], Lai and Lin, unpublished data). The zyxin family proteins are present at cell-matrix adhesion sites and co-localize with the actin cytoskeleton, suggesting that the function of these family members is involved in the regulation of cell adhesion and motility [40]. Moreover, the zyxin family proteins are also demonstrated to shuttle between the cytosol and nucleus, implicating their function in nuclear activities such as transcriptional regulation [52, 54-57].

TRIP6 and Transcriptional Regulation

TRIP6 is capable of shuttling from the cytosol to the nucleus. Mutation or deletion of the NES of TRIP6, or treatment of cells with leptomycin B results in nuclear accumulation of the TRIP6 protein [53, 54]. Several studies have shown that TRIP6 does not bind to DNA directly, but can act as a coactivator of some transcription factors. For example, a fusion protein of LexA and the carboxyl-terminal region of TRIP6 can increase transcriptional activity of LexA-β-galactosidase [39]. Similarly, GAL4-TRIP6 fusion protein activates transcription in yeast and chicken cells [54], and enhances the
transcription of NF-κB-reporter gene by v-Rel [58]. Since TRIP6 interacts with v-Rel, it has been implicated to function as a co-activator of v-Rel.

In addition to serving as a coactivator of transcription factors, TRIP6 also interacts with RIP2 and enhances the ability of RIP2 to transactivate NF-κB by TNF treatment [44], and potentiates RIP2-mediated ERK activation. Moreover, TRIP6 has been shown to interact with TRAF2, TRAF6, IL-1 receptors, toll-like receptor 2 (TLR2), and Nod1, and promote NF-κB activation and ERK activation by IL-1, TLR2 or Nod1. Recently, TRIP6 has been shown to interact with AMPK through it LIM2 and 3 domains [46]. AMPK phosphorylates TRIP6 and increases the ability of TRIP6 to activate NF-κB [46]. Although TRIP6, in itself, can transactivate AP-1 and NF-κB, TRIP6 interacts with glucocorticoid receptor and transrepresses AP-1 and NF-κB activation [45].

**TRIP6 and Cell Motility**

Similar to other zyxin family members, TRIP6 is involved in cell adhesion and motility. Both TRIP6 and zyxin have been shown to interact with p130Cas(Crk-associated substrate), and its family member, CasL/HEF1 [58]. The adaptor protein p130Cas is recruited to integrin-rich sites upon cell-matrix adhesion, and binds to several signaling molecules including Src, Crk (chicken tumor virus 10 regulator of kinase), and FAK (focal adhesion kinase) [59-61]. Overexpressing p130Cas enhances cell migration; in contrast, disruption of p130Cas gene impairs actin stress fiber formation [62].

In addition, TRIP6 has been identified as the binding partner of the protein tyrosine phosphatase PTP1E (also known as PTPL1, which will be used throughout this paper, FAP-1, and PTP-BL mouse homologue)[40, 63-65]. PTPL1 is a non-transmembrane
protein tyrosine phosphatase, which contains multiple protein-protein interacting domains
including kinase noncatalytic C lobe domain (KIND), FERM domain (an acronym of “band
4.1, ezrin, radixin, and moesin”), five PDZ domains, and a catalytic domain in the most
carboxy- terminus[63, 66, 67]. Several actin-associated proteins interact with PTPL1.
For example, adenomatous polyposis coli (APC) protein interacts with the second PDZ
domain of PTPL1[68]. One important function of APC is to regulate β-catenin in cell
adhesion process[69, 70]. APC is also shown to interact with Rho exchange factor (Asef)
and is involved in the regulation of tubulin and actin cytoskeleton[71]. In addition to
APC, PTPL1 also binds to protein kinase C-related kinase-2 (PRK2)[72], which regulates
the small G-protein Rho, and modulates the actin cytoskeleton[73]. Moreover, PTPL1
directly interacts with PARG-1 (PTPL1-associated Rho GTPase activating protein 1)
through its PDZ4 domain[74]. These observatio ns suggest that the function of PTPL1 is
involved in actin dynamics.

TRIP6 was also identified as an endoglin binding protein in a yeast two-hybrid
screen. Endoglin is a component of the TGF-β receptor complex that is expressed at
the surface of endothelial cells. Suppression of endoglin or TRIP6 expression reduces
actin bundle formation [47].

Recently, TRIP6 was shown to interact with a peripheral membrane protein, su-
pervillin, which binds to myosin II and F-actin, and negatively regulates actin stress fiber
formation. Knockdown of supervillin expression results in increased cell adhesion to fi-
bronectin. TRIP6 is implicated in the functional regulation of supervillin in cell adhesion
[75]. In summary, TRIP6 localizes in cell focal adhesions and interacts with multiple
proteins involved in cell cytoskeleton dynamics and cell motility, suggesting an important
and physiological role of TRIP6 in cell motility.

Other Functions of TRIP6

TRIP6 has been implicated in the pathogenesis of certain microorganisms. TRIP6
interacts with the opacity-associated protein (OpaP) of *Neisseria gonorrhoeae*, although
the mechanism remains unclear [41]. In addition, SrfH/SseI (SsrB regulated factor
H/ *Salmonella* secreted effector I), which is involved in type III secreting system, also in-
teracts with TRIP6 and increases cell migration ability [76]. *Salmonella typhimurium-
infected phagocytes penetrate gastrointestinal (GI) epithelium and enter the blood stream
by manipulating SrfH, which may interact with TRIP6 and regulate bacterial motility. *Salmonella* may enhance its systemic spreading through this interaction and accelerate
cell motility in the bloodstream.

Taken together, TRIP6 plays a multifunctional role in different cellular responses.
Here we identify TRIP6 as an LPA$_2$-interacting protein, and demonstrate its regulation in
LPA-induced cell migration.
CHAPTER 2

TRIP6 ENHANCES LY SOPHOSPHATIDIC ACID-INDUCED CELL MIGRATION
BY INTERACTING WITH THE LY SOPHOSPHATIDIC ACID 2 RECEPTOR

by

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CHAPTER 2

TRIP6 ENHANCES LYSOPHOSPHATIDIC ACID-INDUCED CELL MIGRATION BY INTERACTING WITH THE LYSOPHOSPHATIDIC ACID 2 RECEPTOR

Abstract

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)/ZRP-1 (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.

Introduction

Lysophosphatidic acid (LPA) is a bioactive growth factor-like phospholipid, which mediate diverse biological responses such as mitogenesis, differentiation, cell survival, angiogenesis, inflammation, and cell migration (1). Although the functions of LPA were recognized in the mid-1980s, its associated receptors have just been cloned and characterized in the past few years (1). The first three LPA receptors that have been identified belong to the membrane-bound G protein-coupled receptors, including the LPA1/EDG2, LPA2/EDG4, and LPA3/EDG7 receptors of the endothelial differentiation gene family (2–4). Most recently, the G protein-coupled orphan receptor, p2y9/GPR23, has been recognized as the fourth LPA receptor, which is structurally distinct from the other LPA receptors (5). These membrane-bound LPA receptors couple to Gq, Gi/o, or G12/13 proteins and share similar functions in mediating LPA actions (1). Intriguingly, LPA has recently been identified as an agonist of the nuclear peroxisome proliferator-activated receptor γ (6). Thus, some of the LPA signaling pathways are probably differentially regulated by different LPA receptors.

LPA modulates cell adhesion and migration in many cell types by inducing actin cytoskeletal rearrangement, the assembly of focal complexes, and the formation of focal adhesions through a Rho-dependent, integrin-mediated signaling pathway (7, 8). Reciprocal activation of Rho and Rac coordinates the dynamic processes of cell migration (9). The assembly of focal complexes requires focal adhesion kinase (FAK), Src family
kinases, paxillin, and p130cas (Crk-associated substrate) (10). These proteins form complexes with downstream signaling molecules, Grb2 and Crk, and trigger adhesion-induced cellular responses including mitogenic signaling, cell locomotion, and cell survival (11). Thus far, the detailed mechanisms by which LPA receptors mediate LPA-induced cell migration are not clear and remain to be explored.

Recently, members of the zyxin family have been shown to localize at focal adhesions and associate with the Cas family, p130cas and CasL/HEF1 (12). The zyxin family members, including zyxin, LPP (lipoma preferred partner), and TRIP6/ZRP-1, contain three zinc finger LIM domains at their carboxyl terminus, a proline-rich region, and nuclear export signals at their N terminus (12–15). The LIM domain (named by the initials of three homeodomain proteins, Lin-11, Isl-1, and Mec-3) has been demonstrated to be a protein-protein interaction motif that is critically involved in their functions (16). Zyxin has been shown to associate with the actin cytoskeleton and is postulated to function in integrin-mediated signaling (17). These zyxin family members localize at focal adhesions but may shuttle between plasma membrane, cytosol, and nucleus and relay unidentified signals between focal adhesions and nucleus (18–20). Since zyxin and TRIP6 associate with Cas family members, they may cooperate to regulate cell motility (12).

The LPA1, LPA2, and LPA3 receptors share high homology in amino acid sequences except for the carboxyl-terminal region, suggesting that the cytoplasmic tail of these receptors may specifically regulate their functions in LPA signaling. In an attempt to identify the molecules that specifically involve in the function and regulation of the LPA2 receptor, we used the carboxyl-terminal tail of the LPA2 receptor as the bait in a yeast two-hybrid screening. Here we demonstrate that the LPA2 receptor, but not LPA1
or LPA3 receptor, associates with TRIP6 by LPA stimulation. The LPA-dependent recruitment of TRIP6 to the plasma membrane promotes its targeting to focal adhesions and co-localization with actin. TRIP6 then serves as an adaptor for the assembly of focal complexes, thereby regulating LPA-induced cell migration.

**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 sequences of the LPA2 receptor, which was found deleted in the I.M. A.G.E. clone 755526 (21, 22), has been corrected by PCR. The full-length LPA3 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 LPA1–3 receptors, a LPA2 receptor mutant lacking the carboxyl-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 pHcRed1 (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 (HcRed1) at their N termini. The entire sequences of each cDNA clone were verified by automatic DNA sequencing. For *in vitro* binding between TRIP6 and the LPA receptor, cDNA fragments encoding TRIP6 or the cytoplasmic
tail of the LPA2 receptor (aa 296–351) (designated LPA2R-CT) 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 pSUPERsiTRIP6) in mammalian cells, which specifically targets the 19-nt sequences of TRIP6, 5’-GAAGCTGGTTCACGACATG-3’.

**Yeast Two-hybrid Screening**

For the yeast two-hybrid screening, a cDNA fragment encoding LPA2R-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 LPA2R-CT were verified by automatic DNA sequencing. This pASLPA 2R-CT was used as the bait to screen a HeLa cell cDNA library (cDNA constructed in pGAD GH) (Clontech). After co-transformation of pAS-LPA2R-CT and the library plasmids into yeast strain Y190, cells were screened on the plates lacking tryptophan, leucine and histidine supplemented with 25 mM 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 on M9 plates. The positive cDNA clones were sequenced. To construct pAS-LPA3R-CT, a cDNA fragment encoding LPA3R-CT (aa 294–353) was amplified by PCR, inserted into pAS 2-1, and verified by DNA sequencing. To construct pAS-LPA1R-CT, an EcoRI/SalI cDNA fragment encoding amino
acids 301–364 of the LPA1 receptor was removed from pCMV-FLAG-LPA2R 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 (BSA)-containing 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 an anti-GFP or anti-Myc 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-Tag3A or pCMV-Myc-LPA2R. After treatment or not with 2 µM LPA for 10 min, Myc-LPA2R from the whole cell lysates was im-
munoprecipitated 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 LPA2R-CT in vitro, GST, GST-LPA2R-CT, 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-LPA2R-CT in the binding buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) for 3 h at 4 °C. TRIP6 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 PET 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(ΔC1) (aa 1–278), or GFPTRIP6(ΔC2) (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 by fluorescence microscopy (Axioplan 2; Zeiss). Meanwhile, an aliquot of cells was plated on the coverslips. GFP-positive cells in each field 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 LPA2 receptor, SKOV3 cells were transiently co-transfected with the plasmids expressing HcRed1-TRIP6 and GFP-LPA2R. Cells were starved in Dulbecco’s modified Eagle’s medium containing 0.1% BSA overnight and then treated without or with 2 μM LPA for 10 min. The images of GFP-LPA2R and HcRed1-TRIP6 were directly captured by fluorescence microscopy (Axioplan 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 without or with 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), or 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 Carboxyl-terminal Tail of the LPA2 Receptor, but Not LPA1 or LPA3 Receptor, 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 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 sequences of TRIP6 (aa 220–476 and 308–476) were isolated (Fig. 1A). These two clones, designated TRIP6(ΔN1) and TRIP6(ΔN2), encode the carboxyl LIM domains 1–3 and LIM domains 2 and 3 of TRIP6, respectively. The interaction was further verified by selective
growth of yeast cells co-expressing both LPA2R-CT and TRIP6(ΔN1) (Fig. 1B). We fur-
ther examined the ability of the carboxyl-terminal tails of LPA1 and LPA3 receptors to
bind to TRIP6. Strikingly, TRIP6 only interacted with the LPA2 receptor but not LPA1
or LPA3 receptor in yeast (Fig. 1B).

Next, we examined whether TRIP6 directly interacts with the LPA2 receptor. In
this experiment, a GST fusion protein of LPA2R-CT was used to pull down the purified
full-length TRIP6 in vitro. As shown in Fig. 2, TRIP6 was pulled down by GST-LPA2R-
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 re-
ceptor or a LPA2 receptor mutant lacking the cytoplasmic tail (designated LPA2R(ΔC))
was performed in HEK 293T cells, which express a barely detectable level of endogenous
TRIP6. As shown in Fig. 3A, LPA stimulation for 10 min promoted co-
immunoprecipitation of TRIP6 with the LPA2 receptor but not LPA1 or LPA3 receptor.
In contrast, deletion of the carboxyl-terminal tail of LPA2R abolished its interaction with
TRIP6 (Fig. 3A). We then performed co-immunoprecipitation of a Myc-tagged LPA2 re-
ceptor with endogenous TRIP6 in NIH/3T3 fibroblasts. The result showed that endoge-
nous TRIP6 associated with the transfected Myc-tagged LPA2 receptor in a LPA-
dependent manner (Fig. 3B). Taken together from cellular co-immunoprecipitation, yeast
two-hybrid assays and in vitro GST pull-down experiments, it is clear that TRIP6 specifi-
cally 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) 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. Different 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 LPA2R binding ability of different TRIP6 mutants is summarized in Fig. 4E.

LPA Induces Co-localization of TRIP6 with the LPA2 Receptor and Promotes TRIP6 Targeting to Focal Adhesions
To investigate the potential functional role of TRIP6 in LPA2 receptor-mediated signaling, localization of TRIP6 with the LPA2 receptor was first examined in SKOV3 ovarian cancer cells expressing a GFP fusion protein of the LPA2 receptor and a HcRed1 fusion protein of TRIP6. The protein distribution was visualized directly by fluorescence microscopy. As shown in Fig. 4A, GFP-LPA2R was diffusely present or formed small clusters on the plasma membrane and in the cytoplasm by serum starvation overnight. Similarly, HcRed1-TRIP6 was predominantly cytosolic with some aggregates in the un-stimulated cells. However, the distribution of these TRIP6 aggregates did not co-localize with the LPA2R clusters. After exposure of cells to LPA for 10 min, a large portion of HcRed1-TRIP6 appeared in plasma membrane clusters that coincided precisely with the distribution of GFP-LPA2R, indicative of LPA-dependent translocation of TRIP6 to the receptor (Fig. 4A, middle panel). Strikingly, LPA-induced cell rounding was greatly enhanced by overexpression of the LPA2 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.

The LIM domain-containing proteins, including the family members of paxillin and zyxin, have been shown to localize at focal adhesions mainly through the LIM domains; however, the non-LIM region also has some weak targeting capacity (19, 25, 26).
To further investigate LPA-dependent targeting of TRIP6 to focal adhesions, co-localization of TRIP6 with the focal adhesion molecules, including vinculin, FAK, and paxillin, were examined in SKOV3 cells expressing GFP-TRIP6 in the absence or presence of LPA. These SKOV3 cells have been shown to express endogenous LPA1, LPA2, and LPA3 receptors at high levels (27, 28). As shown in Fig. 4B, GFP fluorescence of TRIP6 was diffusely present in the cytoplasm after serum starvation overnight, although a small amount of TRIP6 was found at focal adhesions. After LPA stimulation for 15 min, a large portion of TRIP6 was depleted from the cytosol and recruited to focal adhesions at the periphery of the plasma membrane, where it was co-localized with vinculin (Fig. 4B), FAK (Fig. 4C), and paxillin (Fig. 4D).

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. We found that the focal adhesion targeting capacity of TRIP6 was retained when LIM2 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 membrane-bound TRIP6 might dissociate from the LPA2 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 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, p130cas, 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 receptor-mediated signaling to the assembly of focal complexes and cell migration. The components of focal complexes known to be involved in cell migration include p130cas, FAK, paxillin, and c-Src. To assess whether TRIP6 associates with these molecules by LPA stimulation, we examined LPA-dependent co-immunoprecipitation between TRIP6 and paxillin, p130cas, FAK, or c-Src in HEK 293T cells. Our results showed that LPA induced co-immunoprecipitation of TRIP6 with paxillin (Fig. 6A), p130cas (Fig. 6B), Myc-FAK (Fig. 6C), and HA-c-Src (Fig. 6D). Thus, LPA-promoted recruitment of TRIP6 to the plasma membrane transforms it into an adaptor for the formation of multiple protein complexes. These findings further suggest a potential regulatory role for TRIP6 in LPA-induced cell migration and downstream signaling events.

**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 praline-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 GFPTRIP6(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 pSUPERsiTRIP6 (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 for 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 CasL/HEF1; 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 LPA1 receptor couples to a Gi-phosphoinositide 3-kinase-Tiam1 pathway to activate Rac, and thereby stimulates cell motility (32). Another report demonstrates 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 LPA1 or LPA3 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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References


FIG. 1. TRIP6 interacts with the LPA2 receptor, but not LPA1 or LPA3 receptor in yeast. 
A, the schematic structures of TRIP6 and two TRIP6(ΔN) clones identified as LPA2 receptor-interacting proteins in a yeast two-hybrid screening. TRIP6 contains a nuclear export signal (aa 100–107), a N-terminal proline-rich region, and three C-terminal LIM domains. TRIP6(ΔN1) (aa 220–476) and TRIP6(ΔN2) (aa 308–476) contain LIM domains 1–3 and LIM domains 2 and 3, respectively. 
B, interaction of TRIP6(ΔN1) with LPA2R-CT in yeast. The cDNA fragments encoding the carboxyl-terminal tail of LPA1R (aa 301–364), LPA2R (aa 296–351), and LPA3R (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 LPA2R-CT was verified by selective growth of transformants on a plate lacking tryptophan, leucine, and histidine supplemented with 3-amino-1,2,4-triazole.
FIG. 2. TRIP6 interacts with the carboxyl-terminal tail of the LPA2 receptor in vitro. One µg of purified TRIP6 was incubated with 1 µg of GST or GST-LPA2R-CT (aa 296–351) as described under “Experimental Procedures.” TRIP6 pulled down by GST-LPA2R-CT was detected by immunoblotting (IB) with an anti-TRIP6 antibody. GST-LPA 2R and GST were visualized by Ponceau S staining. The third lane shown is a 10% input of TRIP6 used in this experiment.
FIG. 3. The LIM domains of TRIP6 bind to the LPA2 receptor, but not LPA1R, LPA3R, or a LPA2R mutant lacking its carboxyl-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 LPA2R(ΔC) 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, LPA-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 FLAGLPA2R with GFP-TRIP6(CT2) (aa 339–476) or GFP-TRIP6(CT3) (aa 398–476), which contain LIM domains 2 and 3 and LIM3 domain, re-
spectively. 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 1–278) 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.
FIG. 4. LPA promotes co-localization of TRIP6 with the LPA2 receptor and TRIP6 targeting to focal adhesions. A, SKOV3 cells were co-transfected with the expression plasmids of GFP-LPA2R and HcRed1-TRIP6. After serum starvation overnight, cells were treated without or with 2 µM LPA for 10 min. The GFP fluorescence of LPA2R 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 LPA2 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.
FIG. 5. TRIP6 co-localizes with actin by LPA treatment. A, NIH/3T3 fibroblasts transiently expressing GFP-TRIP6 were treated without or with 2 µM LPA for 20 min. Actin was stained with TRITC-phalloidin. B, the molecular structures of different TRIP6 truncation mutants and a summary of their capacity to co-localize with actin. C, NIH/3T3 fibroblasts expressing different GFP-TRIP6 truncation mutants were treated with 2 µM LPA for 20 min. The top panel shows GFP fluorescence of different TRIP6 mutants, and the bottom panel shows actin stained with TRITC-phalloidin.
FIG. 6. LPA induces the association of TRIP6 with paxillin, p130cas, FAK, and c-Src in HEK 293T cells. A and B, HEK 293T cells were transiently transfected with an empty vector or a Myc-TRIP6-expressing vector. Cells were starved overnight and then treated without or with 2 µM LPA for 10 min. TRIP6 from the whole cell lysates was immuno-precipitated with anti-Myc 9E10 antibody-conjugated agarose beads and resolved by SDS-PAGE. The endogenous paxillin (A) and p130cas (B) co-immunoprecipitated with Myc-TRIP6 were detected with their specific antibodies. C, HEK 293T cells transiently expressing GFP-TRIP6 without or with Myc-FAK were incubated in the absence or presence of 2 µM LPA for 10 min. Myc-FAK was immunoprecipitated (IP) with anti-Myc 9E10 antibody-conjugated agarose beads. The immunoblot (IB) was probed with an anti-GFP antibody to detect GFP-TRIP6. D, LPA-dependent co-immunoprecipitation of HA-c-Src and FLAG-TRIP6 was performed in HEK 293T cells as described above. TRIP6 was immunoprecipitated with anti-FLAG M2 antibody-conjugated agarose beads, and the co-immunoprecipitated c-Src was detected with an anti-HA antibody. The bottom panel of each figure is an immunoblot showing the expression of endogenous paxillin (A), endogenous p130cas (B), transfected GFP-TRIP6 (C), and HA-c-Src (D) in the whole cell lysates.
FIG. 7. TRIP6 enhances LPA-induced but not thrombin-induced cell migration, whereas suppression of endogenous TRIP6 expression by a TRIP6-specific siRNA inhibits it in SKOV3 cells. A, SKOV3 cells transiently expressing GFP, GFP-TRIP6, GFP-TRIP6(CT3), GFP-TRIP6(AΔC1), or GFP-TRIP6(AΔC2) were subjected to haptotactic cell migration assays as described under “Experimental Procedures.” Two µM LPA was added or not in the lower chamber of the transwells, and cells were allowed to migrate for
6 h. Nonmigrated cells from the top surface were removed with a Q-tip. The filter was fixed, and GFP-positive cells migrated to the bottom surface were counted by fluorescence microscopy. LPA-induced cell migration was determined as the -fold increase of migrated cells with LPA exposure versus without LPA exposure. The bottom panel shows images of some migrated cells. B, thrombin-induced cell migration was performed in SKOV3 cells transiently expressing GFP or GFP-TRIP6 as described above. 1 units/ml of thrombin was added or not in the bottom chamber of the transwells. C, pEGFP was transiently co-transfected with pSUPER or pSUPER-siTRIP6 into SKOV3 cells. An LPA-induced haptotactic cell migration assay was carried out as described above. In the meantime, equal amounts of cells were harvested and dissolved in SDS lysis buffer. The bottom panel is an immunoblot showing the expression of endogenous TRIP6 in cells transfected with pSUPER (lane 1) or pSUPER-siTRIP6 (lane 2). This blot was reprobed with an anti-GFP antibody to ensure equal expression of GFP in each sample. Data shown in each figure are the mean ± S.E. of 3–7 independent experiments.
CHAPTER 3

C-SRC-MEDIATED PHOSPHORYLATION OF TRIP6 REGULATES ITS FUNCTION
IN LYSPHOSPHATIDIC ACID-INDUCED CELL MIGRATION

by

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CHAPTER 3
C-SRC-MEDIATED PHOSPHORYLATION OF TRIP6 REGULATES ITS FUNCTION IN LYSOPOPHATIDIC ACID-INDUCED CELL MIGRATION

Abstract

TRIP6 (thyroid receptor-interacting protein 6), also known as ZRP-1 (zyxin-related protein 1), is a member of the zyxin family that has been implicated in cell motility. Previously we have shown that TRIP6 binds to the LPA2 receptor and associates with several components of focal complexes in an agonist-dependent manner and thus, enhances lysophosphatidic acid (LPA)-induced cell migration. Here we further report that the function of TRIP6 in LPA signaling is regulated by c-Src-mediated phosphorylation of TRIP6 at the Tyr-55 residue. LPA stimulation induces tyrosine phosphorylation of endogenous TRIP6 in NIH 3T3 cells and c-Src-expressing fibroblasts, which is virtually eliminated in Src-null fibroblasts. Strikingly, both phosphotyrosine-55 and proline-58 residues of TRIP6 are required for Crk binding in vitro and in cells. Mutation of Tyr-55 to Phe does not alter the ability of TRIP6 to localize at focal adhesions or associate with actin. However, it abolishes the association of TRIP6 with Crk and p130cas in cells and significantly reduces the function of TRIP6 to promote LPA-induced ERK activation. Ultimately, these signaling events control TRIP6 function in promoting LPA induced morphological changes and cell migration.

Introduction
Lysophosphatidic acid (LPA) associates with the G protein coupled LPA receptors and induces actin rearrangement, focal adhesion assembly, and cell migration through a Rho-dependent, integrin-mediated signaling pathway (24). Similar to several other ligands for G protein-coupled receptors, LPA induces a transient increase of Src kinase activity (21) and rapid tyrosine phosphorylation of a number of proteins involved in cell adhesion and migration, such as FAK, p130cas, and paxillin (31). More and more evidence has shown that tyrosine phosphorylation of these signaling molecules plays a critical role in recruiting active signaling molecules into multiprotein complexes (23). Subsequently, these activated proteins coordinately regulate cell adhesion, migration, and downstream signaling events involved in cell proliferation, survival, and apoptosis (5). Using cells from knockout mice and advanced imaging technology, several tyrosine kinases and phosphatases have been demonstrated to modulate the dynamic processes of cell adhesion and migration. For example, FAK-null cells and SYF cells lacking c-Src, Fyn, and Yes of the Src family kinases show reduced cell migration (13, 17). Similarly, overexpression of the protein tyrosine phosphatase PTP-PEST, a phosphatase for tyrosine-phosphorylated p130cas, leads to defective cell migration (1). Moreover, tyrosine phosphorylation of paxillin and p130cas and the activation of extracellular signal-regulated kinases (ERKs) and MLCK (myosin light-chain kinase) have been implicated in FAK/Src-mediated adhesion turnover and disassembly (37). Thus, these signaling molecules utilize tyrosine phosphorylation as a mechanism to coordinately regulate cell adhesion and migration.

TRIP6 (thyroid receptor-interacting protein 6), also known as ZRP-1 (zyxin-related protein 1), is a member of the zyxin family that has been implicated in the regula-
tion of actin dynamics and signal transduction involved in cell adhesion and migration (22, 40, 42). Originally discovered as an interacting protein of the nuclear thyroid hormone receptor in a yeast two-hybrid system (18), TRIP6 was later identified as a focal adhesion molecule with the capability to shuttle between cell surface and nucleus (36). The zyxin family members, including zyxin, TRIP6, LPP (lipoma preferred partner), and Ajuba, possess a proline-rich region and nuclear export signals at their N terminus and three LIM domains at their carboxyl terminus (3, 14, 22, 26). The LIM domains (named by the initials of three homeodomain proteins, Lin-11, Isl-1, and Mec-3) contain two cysteine-rich zinc finger motifs, which are critical for protein-protein interaction (2). Several LIM domain-containing proteins, such as the zyxin family members, paxillin, and other related proteins, have been shown to localize at focal adhesion plaques and associate with actin cytoskeleton (9, 19, 28, 40). They serve as scaffold or adaptor proteins for the assembly of multiple protein complexes involved in actin rearrangement, cell adhesion, and motility.

Previously it has been shown that zyxin and TRIP6 associate with the Cas family members, including p130cas and CasL/HEF1, and may cooperate in cell motility (42). Very recently, we have demonstrated that LPA stimulation promotes the recruitment of TRIP6 to the activated LPA2 receptor and induces the association of TRIP6 with the components of focal complexes, including paxillin, p130cas, FAK, and c-Src (40). Over-expression of TRIP6 enhances LPA-induced cell migration; in contrast, suppression of the endogenous TRIP6 expression by its specific small interfering RNA (siRNA) inhibits it in SKOV-3 ovarian cancer cells (40). Similarly, another zyxin family member, LPP, has been reported to promote epidermal growth factor-induced cell migration in vascular
smooth muscle cells (11). In contrast to zyxin knockout mice, which do not exhibit significant phenotypes (12), the Ajuba-null cells show a reduction of tyrosine phosphorylation of p130cas, Crk, and Dock180 at nascent focal complexes, which leads to the inhibition of Rac activation and impaired cell migration (27). All of these results suggest that these zyxin family members are involved in cell motility. However, the detailed mechanism(s) by which these zyxin family members regulate cell migration is not yet clear.

Despite all of the data suggesting a role for TRIP6 in LPA-induced cell migration, very little is known about how TRIP6 function is regulated in this dynamic process. In the present study, we demonstrate that c-Src mediates LPA-induced TRIP6 phosphorylation at the Tyr-55 residue. This phosphorylation does not regulate its focal adhesion targeting or association with actin cytoskeleton. However, it is critical for the recruitment of Crk and p130cas and for c-Src-mediated ERK activation. Ultimately, these signaling events regulate the process of LPA-induced morphological changes and cell migration.

Materials and Methods

Plasmid construction and site-directed mutagenesis

To express recombinant glutathione S-transferase (GST) fusion proteins of TRIP6-Y55F and TRIP6- P58A mutants in Escherichia coli, site-directed mutagenesis (Promega) was performed using pGEX-6P-3-TRIP6 (40) as the template. The nucleotides TAC encoding Tyr-55 and nucleotides CCA encoding Pro-58 were replaced with TTC and GCG, respectively. The cDNA sequences were verified by automatic DNA sequencing. These mutant cDNA sequences were then inserted into pCMVTag2A, pCMV-Tag3A (Stratagene), and pEGFP-C1 (Clontech), respectively, such that they were tagged in
frame with a FLAG epitope, a MYC epitope, or a green fluorescent protein (GFP) at its N terminus. The pSUPER vector (6) was used to direct the expression of an siRNA of mouse TRIP6 (designated pSUPER-siTRIP6), which specifically targets the 19-nucleotide sequences of mouse TRIP6, 5’-GAAACTGGTGCACTGACATG-3’. The clone containing full-length cDNA sequences of Crk I was purchased from the IMAGE Consortium, amplified by PCR, and inserted into pEGFP-C1, pCMV-Tag2B, and pGEX-6P-3, respectively. The entire sequences were verified by automatic DNA sequencing.

Phosphorylation of TRIP6 by c-Src in vitro and in cells

pGEX-6P-3-TRIP6 was transformed into *E. coli* BL21(DE3)(LysS). GST-TRIP6 was purified and further digested with PreScission protease (Amersham Biosciences) to cleave GST. Purified TRIP6 was phosphorylated by recombinant p60c-Src (Upstate Biotechnology Inc.) at 30°C for 30 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel slices containing phosphorylated TRIP6 were cut out and digested in situ with trypsin. After purification, the phosphorylation sites of TRIP6 were identified by mass spectrometry analysis (Protein Chemistry Core Facility, Baylor College of Medicine). Similarly, 1 µg of TRIP6 or TRIP6-Y55F was purified and incubated with recombinant p60c-Src. The phosphoproteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and detected with a horseradish peroxidase (HRP)-conjugated antiphosphotyrosine antibody (PY20H; Santa Cruz Biotechnology).

To detect tyrosine phosphorylation of TRIP6 in cells, NIH 3T3 cells, Src-null SYF cells, or c-Src-reconstituted SYF+c-Src cells (American Type Culture Collection)
were transiently transfected with an empty vector or the expression vector of TRIP6 or TRIP6-Y55F. Cells were starved in 0.1% fatty acid-free bovine serum albumin (BSA)-containing Dulbecco’s modified Eagle’s medium (DMEM) for 8 h or overnight. Cells were incubated without or with 1 or 2 µM LPA for various times and harvested in lysis buffer (1% Triton X, 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 endogenous or epitope-tagged TRIP6 was immunoprecipitated with a TRIP6-specific monoclonal antibody (Transduction Laboratories) or epitope-specific antibodies and resolved by SDS-PAGE. The tyrosine-phosphorylated TRIP6 was detected by immunoblotting using an HRP-conjugated antiphosphotyrosine antibody (RC20H [Transduction Laboratories] or PY20H [Santa Cruz Biotechnology]).

In vitro binding of Crk I with c-Src-phosphorylated TRIP6

To examine the effect of c-Src-mediated phosphorylation on the binding of TRIP6 with Crk I in vitro, GST, GST-Crk I, GST-TRIP6, GST-TRIP6-Y55F, and GST-TRIP6-P58A were expressed in E. coli and purified by immobilizing the proteins on glutathione-Sepharose 4B beads (Amersham Biosciences). Crk I was further purified by cleaving GST with PreScission protease (Amersham Biosciences). GST and GST fusion proteins of TRIP6, TRIP6-Y55F, and TRIP6-P58A were phosphorylated by c-Src in vitro. Following reaction, GST fusion protein-immobilized glutathione beads were washed four times to remove c-Src. These beads were then incubated with purified Crk I for 3 h at 4°C. Crk I pulled down by GST fusion proteins was resolved by SDS-PAGE, and the immunoblot was probed with a Crk-specific antibody (Transduction Laboratories).
Cellular coimmunoprecipitation and LPA-induced ERK activation

Cellular coimmunoprecipitation was performed as described previously (40). To determine LPA-induced ERK activation, transfected SYF+c-Src cells were starved overnight and then stimulated with 2 μM LPA for 5 or 10 min. The whole-cell lysates were subjected to SDS-PAGE, and the immunoblot was probed with an anti-phospho-ERK polyclonal antibody (Promega). The blot was then stripped and reprobed with an anti-ERK2 monoclonal antibody (Transduction Laboratories) to ensure equal expression of total ERKs. The MEK1-K97A expression vector (30) was cotransfected or not with pEGFP or pEGFP-TRIP6 into SKOV-3 cells. The expression of MEK1 and MEK1-K97A was detected by probing the immunoblot with a MEK1-specific antibody (Transduction Laboratories). The activation of ERKs was quantitated using NIH Image J software.

Immunocytochemistry and time-lapse imaging of live cells

LPA-promoted colocalization of TRIP6 or TRIP6-Y55F with MYC-FAK, vinculin, paxillin, or actin was performed in SYF+c-Src cells or NIH 3T3 cells as described before (40). For live cell imaging, SYF+c-Src cells or SYF cells expressing different GFP constructs as indicated were plated on glass-bottomed 35-mm tissue culture dishes (MatTek Corp.). After starvation for 2 h in DMEM containing 0.1% fatty acid-free BSA and 10 mM HEPES, pH 7.4, GFP-positive cells were visualized with an Olympus IX70 inverted fluorescence microscope using a 100× objective. Ten micromolar LPA was then added at the center of the plates, and the images of GFP-positive cells were acquired
every 20 or 30 s for 30 to 40 min with a Photometrics 1400 charge-coupled device camera under the control of IPLab software (Scanalytics, Inc.).

Transwell cell migration assays

SKOV-3 cells expressing GFP, GFP-TRIP6, or GFP-TRIP6-Y55F in the absence or presence of MEK1-K97A were subjected to a transwell cell migration assay as described previously (40). The bottom of the transwell membrane was coated with 10 µg/ml fibronectin overnight before the experiment. Cells at a density of 150,000/well were placed in the upper chamber, 2 µM LPA was added or not to the lower chamber, and the cells were allowed to migrate for 6 h. After removing the nonmigrated cells from the top surface, the migrated GFP-positive cells on the whole filter were counted by fluorescence microscopy. Meanwhile, an aliquot of cells was placed on the coverslips, and GFP-positive cells in each field were counted to estimate transfection efficiency. The relative migration rate was defined as the increase of migrated cells compared to migrated GFP control cells in the absence of LPA treatment and has been adjusted by transfection efficiency.

Results

c-Src mediates LPA-induced phosphorylation of TRIP6 at the Tyr-55 residue

To explore whether the function of TRIP6 is regulated by LPA-dependent tyrosine phosphorylation, we first investigated LPA-dependent tyrosine phosphorylation of TRIP6 in NIH 3T3 fibroblasts, which express high levels of TRIP6. Indeed, we found
that LPA stimulation for 15 min promoted tyrosine phosphorylation of endogenous TRIP6 in NIH 3T3 cells (Fig. 1A).

Previously we have demonstrated that LPA promotes the interaction of TRIP6 with two tyrosine kinases involved in cell adhesion and migration, including c-Src and FAK (40). Because TRIP6 contains proline-rich sequences in the N-terminal region that allow it to bind to SH3 domain-containing proteins, such as Src, we speculated that this kinase might be a potential candidate for mediating TRIP6 phosphorylation in cells. Therefore, we compared tyrosine phosphorylation of endogenous TRIP6 in SYF mouse embryonic fibroblasts lacking Src, Fyn, and Yes and SYF+c-Src fibroblasts that have been reconstituted with c-Src. Compared to NIH 3T3 cells, these SYF+c-Src cells express a slightly higher level of c-Src (data not shown). As shown in Fig. 1B, tyrosine phosphorylation of TRIP6 was steadily detected in SYF+c-Src cells but not in Src-deficient SYF cells. It reached the highest level after LPA stimulation for 15 min. This result indicates that Src family kinases are responsible for TRIP6 phosphorylation in vivo.

We then set out to map the relevant sites of TRIP6 phosphorylation. The recombinant TRIP6 purified from *E. coli* was subjected to phosphorylation by the active c-Src kinase in vitro, digested with trypsin, and sequenced. The result revealed that TRIP6 is predominantly phosphorylated by c-Src at Tyr-55 (Fig. 2A). Another minor phosphorylation site of TRIP6 is Tyr-123. However, mutation of Tyr-123 to Phe did not alter c-Src-mediated TRIP6 phosphorylation in vitro or in cells (data not shown).

Next, we generated a TRIP6 mutant, in which Tyr-55 was substituted with Phe, and compared the ability of c-Src to phosphorylate the wild type and a Y55F mutant of TRIP6 in vitro. As shown in Fig. 2B, this single mutation greatly eliminated tyrosine
phosphorylation of TRIP6. To confirm the assignment of Tyr-55 as the physiologically relevant site of TRIP6 phosphorylation, FLAG-tagged wild-type or Y55F mutant TRIP6 was expressed in SYF+c-Src cells, and LPA-dependent tyrosine phosphorylation of these proteins was examined. The result showed that LPA-induced tyrosine phosphorylation of TRIP6 was virtually eliminated by mutation of Tyr-55 to Phe (Fig. 2C), although this mutation did not alter the ability of TRIP6 to associate with c-Src (Fig. 2D). Taken together from these results, we conclude that c-Src mediates LPA-induced TRIP6 phosphorylation at the Tyr-55 residue.

The capability of TRIP6 to target to focal adhesions and colocalize with actin is not affected by Tyr-55 mutation

To investigate how Tyr-55 phosphorylation regulates the function of TRIP6 in LPA signaling, we first examined LPA-dependent recruitment of TRIP6 and the TRIP6-Y55F mutant to focal adhesions and colocalization with actin cytoskeleton. Previously we have shown that TRIP6 is predominantly cytosolic or forms small clusters at sites of adhesions in SKOV-3 cells after serum starvation overnight (40). Similarly, we have found that by serum starvation overnight, both TRIP6 and TRIP6-Y55F were diffusely distributed in the cytoplasm in SYF+c-Src cells or NIH 3T3 cells. Only a small amount of TRIP6 or TRIP6- Y55F colocalized with vinculin, paxillin, and transfected MYC-FAK in SYF+c-Src cells (see Fig. S1A, C, and E in the supplemental material), or with actin in NIH 3T3 cells (see Fig. S1G in the supplemented material). With LPA stimulation for 15 min, substantial amounts of TRIP6 and TRIP6-Y55F have been found to associate with endogenous vinculin, paxillin, and transfected MYC-FAK in SYF+c-Src cells (see Fig.
S1B, D, and F in the supplemental material), and colocalize with actin in NIH 3T3 cells (see Fig. S1H in the supplemental material). Thus, the function of TRIP6 in LPA-induced focal adhesion targeting and actin colocalization is not changed by this point mutation. The localization of vinculin, paxillin, transfected MYC-FAK, and actin in GFP control cells treated with LPA or not is shown in Fig. S2 in the supplemental material.

**Tyr-55 phosphorylation of TRIP6 regulates its binding to Crk and p130cas.**

The majority of Src substrates identified so far contain SH2 or SH3 binding domains, or both, which are important for protein-protein interaction during the organization of signaling pathways. When phosphorylated, the phosphotyrosine motifs bind with a high degree of specificity to SH2 domains or phosphotyrosine binding domains contained in different proteins (25). Several such molecules, including p130cas, paxillin, c-Cbl, and Gab1, utilize pYXXP motifs to bind the Crk SH2 domain (10). Here, phosphotyrosine and proline residues are critical for this interaction. Analogously, the Y55-QA-P motif of human TRIP6 (Fig. 2A) or Y55-Q-P-P motif of mouse TRIP6 may conform to a binding site for the Crk SH2 domain once Tyr-55 is phosphorylated by c-Src. To test this hypothesis, we first examined the importance of the pY55-QA-P58 motif of TRIP6 in Crk I binding in vitro. Thus, GST fusion proteins of wild-type TRIP6, TRIP6-Y55F mutant, and TRIP6-P58A mutant were subjected to in vitro phosphorylation by a recombinant c-Src kinase. Following the reaction, c-Src was removed by extensive washing, and the GST fusion proteins, phosphorylated or not, were used to pull down recombinant Crk I, which contains one SH2 domain and one SH3 domain. As shown in Fig. 3A, c-Src phosphorylated TRIP6 and the TRIP6-P58A mutant equally well, which was greatly
eliminated by Y55F mutation. Strikingly, only the c-Src phosphorylated TRIP6 was able to pull down Crk I, but not nonphosphorylated TRIP6 or the TRIP6-Y55F mutant (Fig. 3B). Although the TRIP6-P58A mutant was phosphorylated by c-Src, it was unable to bind Crk I (Fig. 3B). We further examined the association of TRIP6 with Crk I in HEK 293T cells. In all of the following experiments using GFP constructs to perform coimmunoprecipitation, GFP did not nonspecifically bind to the antibody-conjugated agarose beads (data not shown). As shown in Fig. 4A, LPA promoted the interaction of TRIP6 with Crk I in HEK 293T cells only when c-Src was coexpressed. In contrast to wild-type TRIP6, the TRIP6-Y55F mutant was unable to associate with Crk I in HEK 293T cells even if c-Src was overexpressed (Fig. 4B). Similarly, mutation of Pro-58 to Ala disrupted the interaction of TRIP6 with Crk I in c-Src-expressing HEK 293T cells (see Fig. S3 in the supplemental material). These results indicate that c-Src-mediated phosphorylation of TRIP6 at Tyr-55 renders TRIP6 capable of binding to Crk in vitro and in cells.

Previously it had been shown that LIM domains 1 and 2 of TRIP6 associate with the CAS (Crk-associated substrate) family members, including p130cas and CasL/HEF, in a yeast two-hybrid screen (42). We then demonstrated that LPA stimulation promotes the interaction of TRIP6 with p130cas (40). Because p130cas also binds Crk through Src-dependent phosphorylation at multiple YXXP motifs of substrate domains (5), it is important to examine if TRIP6, Crk, and p130cas are present in a macromolecular complex by LPA stimulation. Indeed, both GFP-Crk I and endogenous p130cas were coimmunoprecipitated by wild-type TRIP6, but not TRIP6-Y55F, in an LPA-dependent manner (Fig. 4B). These results suggest that LPA stimulation promotes the c-Src-dependent complex formation among TRIP6, Crk, and p130cas. The endogenous p130cas could not
be detected in TRIP6-Y55F immunoprecipitates, suggesting that although LIM domains 1 and 2 are responsible for a direct binding of TRIP6 to p130cas, phosphorylation at Tyr-55 by c-Src is also important for TRIP6 to form a stable complex with p130cas. It should be noted that in contrast to our previous finding that LPA induces the association of TRIP6 with p130cas (40), here we were unable to detect endogenous p130cas in the immunoprecipitates of TRIP6 if Crk I was not coexpressed (Fig. 4B, lanes 7 and 8). This result may suggest that the binding affinity between TRIP6 and p130cas is very weak in HEK 293T cells, but the complex formation can be promoted through c-Src-dependent Crk binding. We further examined the effect of TRIP6 on the association of Crk and p130cas in SYF cells and SYF+c-Src cells by overexpressing a TRIP6-specific siRNA. As shown in Fig. S4 in the supplemental material, the endogenous p130cas was present in FLAG-Crk I immunoprecipitates only in SYF+c-Src cells, but not in SYF cells, indicating that c-Src is required for this binding. However, this coimmunoprecipitation was not significantly altered by suppression of TRIP6 expression with a TRIP6-specific siRNA (see Fig. S4, lanes 9 and 10, in the supplemental material), suggesting that the association of Crk and p130cas is not affected by TRIP6.

*LPA-stimulated ERK activation is regulated by tyrosine phosphorylation of TRIP6.*

Many G protein-coupled receptors, including the LPA receptors, mediate Ras-dependent activation of ERK cascades. Substantial evidence has shown that recruitment and activation of the Src family kinases regulate these signaling pathways (20). In addition to mediating mitogenic signaling, activated ERKs have been shown to regulate adhesion turnover and cell migration (34, 37). Phosphorylation of MLCK by the activated
ERK can modulate actomyosin contractility (15) and promote adhesion disassembly (37). Moreover, the activated Gi/o-ERK pathway has been shown to contribute to LPA-induced pancreatic tumor cell migration (33). To examine if TRIP6 is involved in these signaling pathways, we knocked down the expression of endogenous TRIP6 in SYF and SYF+c-Src fibroblasts by a mouse TRIP6-specific siRNA and assessed its effect on LPA-induced ERK activation. As shown in Fig. 5A, the Src-deficient SYF cells exhibited a lower level of LPA-activated ERKs compared to SYF+c-Src cells (top panel, compare lanes 2 and 6). The TRIP6-specific siRNA significantly knocked down the expression of endogenous TRIP6 (third panel) and reduced LPA-induced ERK activation more dramatically in SYF+c-Src cells (top panel, compare lanes 6 and 7 with lanes 9 and 10) than in SYF cells (top panel, compare lanes 2 and 4), suggesting that TRIP6 is involved in c-Src-mediated ERK activation. This effect was apparently regulated by Tyr-55 phosphorylation, since LPA-induced ERK activation was enhanced in SYF+c-Src cells overexpressing TRIP6 but was reduced in that overexpressing TRIP6-Y55F mutant (Fig. 5B).

The function of TRIP6 to promote LPA-induced morphological changes and cell migration is regulated by c-Src-mediated Tyr-55 phosphorylation.

LPA stimulation results in cytoskeletal rearrangement with a concomitant change of cell morphology through the activation of Rho GTPases (24). To explore the role of TRIP6 in LPA-stimulated morphological changes, SYF+c-Src fibroblasts expressing GFP, GFP-TRIP6, or GFP-TRIP6-Y55F were plated on glass-bottomed dishes, starved for 2 h, and then stimulated with LPA. The images of live cells were acquired by time-lapse fluorescence microscopy. As shown in Fig. 6A, soon after LPA stimulation for ~5 to 10 min,
GFP-expressing cells started to retract their cell bodies and completely rounded up after LPA treatment for 22.8 ± 1.0 min (n = 6). Subsequently, they displayed cell surface blebbing and pseudopodium formation. Strikingly, cells expressing GFPTRIP6 exhibited rapid morphological changes and became rounded soon after LPA stimulation for 8.6 ± 1.1 min (n = 9 in three independent experiments; P < 0.01 versus GFP control; Student’s t test). In contrast, cells expressing GFP-TRIP6-Y55F required a much longer time to round up, which could be from 16 to 35 min (29.8 ± 2.8 min; n = 5 in three independent experiments; P < 0.01 versus GFP-TRIP6-expressing cells). Moreover, with the concomitant changes of cell morphology, wild-type TRIP6 at sites of adhesions disappeared much faster than TRIP6-Y55F, as shown in videos S1 and S2 in the supplemental material.

To verify the physiological function of TRIP6 in LPA-induced morphological changes, we further knocked down the expression of endogenous TRIP6 by its specific siRNA. In this experiment, SYF+c-Src cells harboring pEGFP with either pSUPER or pSUPER-siTRIP6 were stimulated with LPA, and time-lapse fluorescence microscopy of the GFP-positive cells was performed. The result showed that cells harboring pEGFP and the pSUPER empty vector completely rounded up after LPA stimulation for 18.7 ± 1.3 min (n = 5); however, suppression of endogenous TRIP6 expression by its specific siRNA slowed the process to 26.3 ± 2.5 min (n = 6 in three independent experiments; P < 0.05 versus pSUPER-expressing cells) (Fig. 6B). All of these findings suggest that TRIP6 is involved in the dynamic process of LPA-induced morphological changes. Moreover, phosphorylation of TRIP6 at Tyr-55 regulates this activity in SYF+c-Src cells.

If c-Src-mediated phosphorylation of TRIP6 is important for its function in LPA-induced morphological changes, expression of wild-type TRIP6 in Src-null SYF cells
would not promote this process. To test this hypothesis, SYF cells expressing GFP or GFP-TRIP6 were stimulated with LPA, and time-lapse imaging was performed. As shown in Fig. 6C, the GFP control SYF cells rounded up soon after LPA stimulation. Compared to the elongated shapes of SYF+c-Src fibroblasts, the morphology of SYF cells was more square-like. Thus, we did not observe a dramatic retraction of cell bodies in these cells. Interestingly, overexpression of TRIP6 in SYF cells did not promote LPA-induced morphological changes as it did in SYF+c-Src cells, and it even inhibited LPA-induced cell rounding. The turnover of TRIP6 at the sites of adhesions seemed much slower in SYF cells than in SYF+c-Src cells. All of these results suggest that c-Src-mediated phosphorylation of TRIP6 regulates LPA-induced morphological changes and possibly affects the turnover of TRIP6 at sites of adhesions as well.

Previously it was shown that coupling of tyrosine-phosphorylated p130cas and the Crk SH2 domain is critical for cell motility (7, 16). Since Tyr-55 mutation disrupts the coupling of TRIP6 to Crk and p130cas and reduces LPA-induced ERK activation, it might affect LPA-induced cell migration. To understand how Tyr-55 mutation affects TRIP6 function in LPA-induced cell migration, GFP, GFP-TRIP6, or the GFP-TRIP6-Y55F mutant was transiently expressed in SKOV-3 cells, and LPA-induced cell migration was assessed. As shown in Fig. 7, in the absence of ligand stimulation, the relative cell migration rate was slightly higher in cells overexpressing GFP-TRIP6 and the GFP-TRIP6-Y55F mutant than the GFP control cells. After LPA stimulation, cell migration was highly increased by overexpression of TRIP6. However, the ability of TRIP6 to promote LPA-induced cell migration was significantly reduced by Tyr-55 mutation. Similar results were observed in NIH 3T3 cells and SYF+c-Src cells (data not shown).
This finding suggests that phosphorylation of TRIP6 at Tyr-55 promotes LPA-induced cell migration. However, the function of TRIP6 in this event is also regulated by another mechanism(s), since TRIP6-Y55F does not act as a dominant-negative mutant.

The role of activated ERK has been shown to play a critical role in LPA-induced migration of human pancreatic cancer cells (33). To investigate if the effect of TRIP6 in promoting LPA-induced cell migration is due to the activation of ERKs or its coupling to Crk and p130cas, or both, we employed a dominant-negative MEK1-K97A mutant to inhibit phosphorylation of ERKs and then examined its effect on TRIP6 function in LPA-stimulated migration of SKOV-3 cells. As shown in Fig. 8, overexpression of the MEK1-K97A mutant was able to inhibit ERK phosphorylation and reduce LPA-induced cell migration in the GFP control SKOV-3 cells. In the absence of LPA, the basal levels of migrated cells were slightly higher in TRIP6-expressing cells, which were not altered by MEK1-K97A. This is not surprising, since the levels of activated ERKs are already very low under serum-free conditions. Interestingly, the effect of TRIP6 in promoting LPA-induced chemotaxis was partially reduced by overexpression of MEK1-K97A. However, MEK1-K97A did not act as a dominant-negative mutant in blocking LPA-induced cell migration. These results suggest that the effect of TRIP6 in promoting LPA-induced cell migration is partly due to the activated ERKs; however, other TRIP6-mediated signaling pathways, such as the recruitment of Crk and p130cas, also contribute to its function in cell migration.

Discussion
Cell migration is a dynamic process that requires a tight coordination of various signaling molecules involved in cell adhesion assembly and disassembly. The fundamental role of FAK-Src signaling pathways in adhesion turnover and cell migration has been demonstrated previously (37, 38). Several substrates for the FAK-Src complex, including paxillin and p130cas, have been shown to be involved in this dynamic process. Analogously, the function of TRIP6 is regulated by c-Src-mediated phosphorylation. It is likely that a coordinated cycling of tyrosine-phosphorylated and –dephosphorylated TRIP6 mediates its association and dissociation with Crk, p130cas, and other adhesion molecules, which in turn regulates the recruitment and turnover of TRIP6 at sites of adhesions. In conjunction with other signaling events, they orchestrate the dynamic process of adhesion formation and turnover and thus control LPA-induced cell rounding and migration.

The zyxin family members share a high degree of sequence homology in the carboxyl-terminal LIM domains; however, the N-terminal proline-rich sequences are much less homologous (22). The Tyr-55 residue is only present in TRIP6 and not other zyxin family members. Thus, they must be regulated either by phosphorylation at other sites or by totally different mechanisms. Whether c-Src-mediated phosphorylation is only specific to TRIP6 but not other zyxin family members remains to be determined. However, our results do reveal some analogies between TRIP6 and another LIM domain-containing focal adhesion molecule, paxillin. First, tyrosine phosphorylation of paxillin is also induced by LPA stimulation (31). Second, this phosphorylation is not required for their localization to focal adhesions; instead, it mediates the recruitment of Crk and promotes their disassembly at the sites of adhesions (35, 37). Consequently, it regulates their functions in cell motility. However, there are also some distinctions between these two mole-
cules. It has been reported that FAK, in association with c-Src, phosphorylates paxillin at Tyr-31 and Tyr-118 (4, 29). Although LPA induces the association of TRIP6 with FAK (40), overexpression of FAK barely increases tyrosine phosphorylation of TRIP6 (data not shown). Instead, TRIP6 is predominantly phosphorylated at Tyr-55 by c-Src (Fig. 2). Previously it was shown that paxillin associates with the protein tyrosine phosphatase PTP-PEST (32). This binding may be necessary for the targeting of PTP-PEST to focal adhesions to facilitate dephosphorylation of the main PTP-PEST substrate, p130cas (1, 8, 32). However, paxillin does not seem to be a direct substrate for PTP-PEST (1). Although it has been shown that the third LIM domain and carboxyl-terminal region of TRIP6 bind to the second PDZ domain of human PTP-1E and its mouse homologue, PTP-BL, the functional significance for this interaction is not known (22). It is also not clear whether TRIP6 is a substrate for PTP-1E or PTP-BL in cells.

In this report, we demonstrate that overexpression of TRIP6, but not the Y55F mutant of TRIP6, promotes LPA-induced morphological changes in SYF+c-Src cells (Fig. 6A). In contrast, overexpression of TRIP6 in SYF cells markedly retards the whole process (Fig. 6C). These results suggest that the effect of TRIP6 in LPA-induced morphological changes is dependent on c-Src-mediated phosphorylation. Since the nontyrosine-phosphorylated TRIP6 is able to target to focal adhesions and colocalize with actin in SYF cells, it is possible that it forms stable complexes with other signaling molecules, which results in more mature adhesions, thereby perturbing adhesion disassembly. Thus, the balance between the expression of phosphorylated and dephosphorylated TRIP6 would be important for normal control of its function in adhesion turnover and cell motility.
The aberrant regulation of Src family kinases has been shown to associate with the initiation and progression of various cancers. For example, in late-stage ovarian cancers in which the levels of LPA are elevated (41), the Src family kinases are also overexpressed and activated (39). Whether LPA-induced tyrosine phosphorylation of TRIP6 is aberrantly regulated by the activated Src family kinases during ovarian cancer progression and whether it contributes to ovarian tumor invasion and metastasis remain to be investigated in the future.

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References


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FIG. 1. LPA induces tyrosine phosphorylation of endogenous TRIP6 in NIH 3T3 and SYF+c-Src cells, but not SYF cells. A. NIH 3T3 cells were starved overnight in DMEM containing 0.1% fatty acid-free BSA. After stimulation with 1 µM LPA or not for 15 min, endogenous TRIP6 in the whole-cell lysates as shown in the bottom panel was immunoprecipitated (IP) with an anti-TRIP6 monoclonal antibody or a mouse immunoglobulin G (IgG). The immunoblot (IB) was probed with an HRP-conjugated antiphosphotyrosine antibody (RC20H). B. Mouse embryonic fibroblasts lacking Src, Fyn, and Yes (SYF cells) or reconstituted with c-Src (SYF+c-Src cells) were starved for 8 h and then treated with LPA for the indicated times. The endogenous TRIP6 was immunoprecipitated with a TRIP6 antibody or a mouse IgG, and the immunoblot was probed with an HRP-conjugated antiphosphotyrosine antibody (PY20H). The bottom two panels show the expression of c-Src and TRIP6 in the whole-cell lysates, respectively.
FIG. 2. TRIP6 is phosphorylated at the Tyr-55 residue by c-Src. A. Tryptic phosphopeptide of TRIP6. Purified TRIP6 was phosphorylated by c-Src in vitro, resolved by SDS-PAGE, and digested with trypsin in situ. After purification, the phosphorylation sites of TRIP6 were identified by mass spectrometry analysis. The phosphorylated Tyr-55 residue is underlined. B. Mutation of Tyr-55 to Phe eliminates phosphorylation of TRIP6 in vitro. Purified wild-type (WT) or Y55F mutant of TRIP6 was phosphorylated by c-Src in vitro. The immunoblot was probed with an HRP-conjugated antiphosphotyrosine antibody (PY20H). Equal loading of purified proteins is shown by Coomassie blue staining. C. LPA-induced TRIP6 phosphorylation is eliminated by mutation of Tyr-55 to Phe. SYF+c-Src cells alone or those expressing FLAG-tagged TRIP6 or TRIP6-Y55F were starved for 8 h and then treated without or with LPA for 15 min. TRIP6 in the whole-cell lysates was immunoprecipitated with the anti-FLAG M2 monoclonal antibody-conjugated agarose beads, and the immunoblot was probed with an HRP-conjugated antiphosphotyrosine antibody (top panel). The blot was then stripped and reprobed with an anti-FLAG polyclonal antibody to detect immunoprecipitated TRIP6 or TRIP6-Y55F (bottom panel). D. Tyr-55 mutation does not affect the association of TRIP6 with c-Src. HEK 293T cells expressing HA-c-Src alone or with either MYC-TRIP6 or MYC-TRIP6-Y55F were starved overnight and then stimulated with LPA for 15 min. TRIP6 in the whole-cell lysates was immunoprecipitated with the anti-MYC monoclonal antibody-conjugated agarose beads. The immunoblot was probed with an anti-HA polyclonal antibody to detect coimmunoprecipitated c-Src (top panel). The blot was stripped and reprobed with an anti-MYC polyclonal antibody to detect immunoprecipitated TRIP6 or TRIP6-Y55F (middle panel). The bottom panel shows the expression of HA-c-Src in the whole-cell lysates.
FIG. 3. Mutation of Tyr-55 or Pro-58 abolishes c-Src-dependent TRIP6 binding to purified Crk I in vitro. A. c-Src phosphorylates wild-type TRIP6 and TRIP6-P58A, but not TRIP6-Y55F, in vitro. Purified TRIP6, TRIP6-Y55F, or TRIP6-P58A was phosphorylated by recombinant c-Src kinase in vitro. The immunoblot was probed with an HRP-conjugated antiphosphotyrosine antibody (top panel). The blot was stripped and reprobed with an anti-TRIP6 antibody (bottom panel). B. GST or GST fusion protein of TRIP6, TRIP6-Y55F, or TRIP6-P58A was phosphorylated by c-Src or not in vitro. Following the reaction, c-Src was removed from GST fusion proteins by washing the beads four times, and these GST fusion proteins were incubated with purified recombinant Crk I. Crk I pulled down by GST fusion proteins was detected with a Crk-specific antibody. The first lane is 10% input of Crk I used in this experiment.
FIG. 4. LPA promotes the association of Crk I and p130cas with TRIP6, but not TRIP6-Y55F, in HEK 293T cells overexpressing c-Src. A. LPA-dependent interaction of Crk I with TRIP6 requires c-Src. GFP-Crk I was expressed without or with FLAG-TRIP6 and hemagglutinin (HA)–c-Src in HEK 293T cells as indicated. Cells were starved overnight and then treated with 2 μM LPA or not for 15 min. TRIP6 in the whole-cell lysates was immunoprecipitated with the anti-FLAG M2 monoclonal antibody-conjugated agarose beads, and the immunoblot was probed with an anti-GFP polyclonal antibody to detect coimmunoprecipitated Crk I (top panel). The bottom three panels show the expression of GFP-Crk I, HA–c-Src, and FLAG-TRIP6 in the wholecell lysates, respectively. B. Crk I and p130cas are present in the immunoprecipitates of TRIP6, but not that of TRIP6-Y55F,
in HEK 293T cells overexpressing HA–c-Src. HA–c-Src was coexpressed with GFP-Crk I or GFP and either MYC-TRIP6 or MYC-TRIP6-Y55F in HEK 293T cells as indicated. Cells were treated with LPA or not as described above. MYC-TRIP6 or MYC-TRIP6-Y55F in the whole-cell lysates was immunoprecipitated with the anti-MYC (9E10) monoclonal antibody-conjugated agarose beads. The immunoblot was detected with an anti-GFP polyclonal antibody followed by a p130cas-specific monoclonal antibody. The blot was then stripped and reprobed with an anti-MYC polyclonal antibody to detect immunoprecipitated TRIP6 or TRIP6-Y55F. The bottom three panels show the expression of GFP-Crk I, GFP, endogenous p130cas, and HA–c-Src in the whole-cell lysates.
FIG. 5. Phosphorylation of TRIP6 at Tyr-55 regulates LPA-induced ERK activation in SYF+c-Src cells. A. Suppression of endogenous TRIP6 expression reduces LPA-induced ERK activation in SYF+c-Src cells. SYF or SYF+c-Src cells were transfected with either pSUPER or pSUPER-siTRIP6. Cells were starved for 8 h and then treated with LPA for 5 or 10 min. Equal amounts of whole-cell lysates were subjected to SDS-PAGE. The immunoblot was probed with an anti-phospho-ERK polyclonal antibody to detect activated ERKs (top panel). The immunoblot was then stripped and reprobed with an anti-ERK2 monoclonal antibody to detect total ERKs (second panel). The third panel shows the expression of TRIP6 in the whole-cell lysates, which was dramatically inhibited by a TRIP6-specific siRNA. The immunoblot shown in the third panel was then stripped and reprobed with an anti-β-actin antibody to ensure equal loading of the proteins (bottom panel). The LPA-stimulated increase of ERK phosphorylation is quantitated and has been adjusted by total ERK expression using NIH Image J software. B. LPA-induced ERK activation is reduced by overexpression of TRIP6-Y55F in SYF+c-Src cells. LPA-induced ERK activation was examined in SYF+c-Src cells expressing GFP, GFP-TRIP6, or GFP-TRIP6-Y55F as described above (top two panels). The immunoblot was probed with an anti-GFP polyclonal antibody to detect the expression of GFP, GFP-TRIP6, and GFP-TRIP6- Y55F in the whole-cell lysates (bottom panel). The activation of ERKs by LPA stimulation is quantitated and has been adjusted by total ERK expression in the whole-cell lysates.
FIG. 6. Phosphorylation of TRIP6 at Tyr-55 regulates LPA-induced morphological changes. A. LPA-stimulated morphological changes are promoted by TRIP6, but not TRIP6-Y55F, in SYF+c-Src cells. SYF+c-Src cells expressing GFP, GFP-TRIP6, or GFP-TRIP6-Y55F were seeded on glass-bottomed 35-mm dishes. After starvation for 2 h,
cells were stimulated with 10 µM LPA and the images of GFP-positive live cells were acquired every 30 s for 30 to 40 min using an inverted fluorescence microscope under the control of IPLab software. Data shown are a comparison of the images captured at various times. B. Suppression of endogenous TRIP6 expression slows the process of LPA-induced morphological changes. A similar experiment as that described for panel A was performed in SYF+c-Src cells harboring pEGFP with either pSUPER or pSUPER-siTRIP6. C. Time-lapse imaging of SYF cells expressing GFP or GFP-TRIP6 was performed as described above. Results shown in panels A to C are representative of three to five independent experiments.
FIG. 7. Tyr-55 phosphorylation of TRIP6 regulates its function in LPA-induced cell migration. SKOV-3 cells were transiently transfected with pEGFP, pEGFP-TRIP6, or pEGFP-TRIP6-Y55F. After washing twice with 1% fatty acid-free BSA-containing DMEM, cells were subjected to transwell cell migration assays. Two micromolar LPA was added or not in the lower chamber of the transwells, and cells were allowed to migrate for 6 h. Nonmigrated cells from the top surface were removed. The filter was fixed, and GFP-positive cells that migrated to the bottom surface were counted by fluorescence microscopy. The relative cell migration rate in each sample is defined as the fold increase of migrated cells compared to the migrated GFP-expressing cells in the absence of LPA stimulation and has been adjusted by transfection efficiency of GFP expression. Data shown are the means ± standard errors of three independent experiments. *, $P < 0.05$ versus LPA-stimulated GFP control cells; **, $P < 0.05$ versus LPA-stimulated GFP-TRIP6-expressing cells (Student’s $t$ test).
FIG. 8. The dominant-negative MEK1-K97A mutant reduces LPA-induced cell migration in SKOV-3 cells expressing GFP or GFP-TRIP6. SKOV-3 cells were transfected with pEGFP or pEGFP-TRIP6 without or with pcDNA3-MEK1-K97A. LPA-stimulated transwell cell migration was performed as described in the legend for Fig. 7. The results shown are the means ± standard errors of four independent experiments. *, $P < 0.05$ versus LPA-stimulated GFP control cells; **, $P < 0.05$ versus LPA-stimulated GFP control cells; ***, $P < 0.05$ versus LPA-stimulated GFP-TRIP6-expressing cells (Student's $t$ test). At the right lower corner is a representative immunoblot showing the expressional levels of phospho-ERKs, total ERKs, and MEK1 with MEK1-K97A in the whole-cell lysates.
FIG. S1. Tyr-55 mutation does not alter the ability of TRIP6 to target to focal adhesions or co-localize with actin by LPA stimulation. SYF+c-Src cells expressing GFP-TRIP6 or GFP-TRIP6-Y55F (A, B), MYC-TRIP6 or MYC-TRIP6-Y55F (C, D), MYC-FAK with GFP-TRIP6 or GFP-TRIP6-Y55F (E, F) were starved for 8 h and then treated with LPA (A, C, E) or not (B, D, F) for 15 min. After fixation of the cells, GFP-TRIP6 and GFP-TRIP6-Y55F were visualized by fluorescence microscopy. The endogenous vinculin (A, and B) and paxillin (C, D) were detected with their specific antibodies followed by a Texas Red X- or a FITC-conjugated secondary antibody, respectively. MYC-TRIP6, MYC-TRIP6-Y55F and MYC-FAK were detected with an anti-MYC (9E10) antibody followed by a Texas Red X-conjugated secondary antibody (E, F). Similarly, NIH/3T3 cells expressing GFP-TRIP6 or GFP-TRIP6-Y55F were starved and then treated with LPA (G) or not (H) for 20 min. Actin was stained with TRITC-phalloidin.
FIG. S2. Subcellular localization of vinculin, paxillin, MYC-FAK in SYF+c-Src cells or actin in NIH/3T3 cells expressing GFP. SYF+c-Src cells or NIH/3T3 cells expressing GFP without or with MYC-FAK were starved for 8h and then treated with LPA or not for 15 min. The localization of vinculin (A), paxillin (B), MYC-FAK (C) or actin (D) was detected as described in Supplemental figure 1.
FIG. S3. Crk I associates with wild-type TRIP6, but not TRIP6-Y55F or TRIP6-P58A mutant, in HEK 293T cells overexpressing c-Src. HEK 293T cells expressing FLAG-Crk I, HA-c-Src and either GFP-TRIP6, GFP-TRIP6-Y55F or GFP-TRIP6-P58A were starved overnight and then stimulated with 2 µM LPA for 15 min. FLAG-Crk I was immunoprecipitated with the anti-FLAG M2 monoclonal antibody-conjugated agarose beads. The immunoblot was probed with an anti-GFP antibody to detect co-immunoprecipitated TRIP6 or mutants (top panel). The blot was stripped and reprobed with an anti-FLAG polyclonal antibody to detect immunoprecipitated FLAG-Crk I (2nd panel). The bottom two panels show equal expression of GFP-TRIP6, GFP-TRIP6-Y55F, GFP-TRIP6-P58A and HA-c-Src in the whole cell lysates.
FIG. S4. Suppression of endogenous TRIP6 expression does not affect c-Src-dependent association of Crk I with p130cas. SYF or SYF+c-Src cells were transfected with a FLAG-Crk I expression vector with either pSUPER or pSUPER-siTRIP6. After starvation for 8h, cells were treated with LPA for 15 min, and Crk I in the whole cell lysates was immunoprecipitated with the anti-FLAG M2 monoclonal antibody-conjugated agarose beads. Co-immunoprecipitated endogenous p130cas was detected by probing the immunoblot with its specific antibody. The blot was stripped and reprobed with an anti-FLAG polyclonal antibody to detect immunoprecipitated Crk I. The bottom two panels show the expression of endogenous p130cas and TRIP6 in the whole cell lysates.
CHAPTER 4

PTPL1/FAP-1 NEGATIVELY REGULATES TRIP6 FUNCTION IN
LYSOPHOSPHATIDIC ACID-INDUCED CELL MIGRATION

by

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Abstract

The LIM domain-containing TRIP6 (Thyroid Hormone Receptor-Interacting Protein 6) is a focal adhesion molecule known to regulate LPA-induced cell migration through the interaction with the LPA2 receptor. LPA stimulation targets TRIP6 to the focal adhesion complexes and promotes c-Src-dependent phosphorylation of TRIP6 at Tyr-55, which creates a docking site for the Crk SH2 domain, thereby promoting LPA-induced morphological changes and cell migration. Here we further demonstrate that a switch from c-Src-mediated phosphorylation to a PTPL1/FAP-1 (Fas-associated phosphatase-1) dependent dephosphorylation serves as an inhibitory feedback control mechanism of TRIP6 function in LPA-induced cell migration. PTPL1 dephosphorylates phosphotyrosine-55 of TRIP6 in vitro and inhibits LPA-induced tyrosine phosphorylation of TRIP6 in cells. This negative regulation can be reversed by suppression of PTPL1 expression or deletion of the LIM3 and PDZ-binding domain of TRIP6 required for PTPL1 interaction. In contrast to c-Src, PTPL1 prevents TRIP6 turnover at the sites of adhesions. As a result, LPA-induced association of TRIP6 with Crk, and the function of TRIP6 to promote LPA-induced morphological changes and cell migration are inhibited by PTPL1. Together, these results reveal a novel mechanism by which PTPL1
phosphatase plays a counteracting role in regulating TRIP6 function in LPA-induced cell migration.

Introduction

The LIM domain-containing TRIP6, also known as ZRP-1 (Zyxin-related Protein 1), is a zyxin family member that has been implicated in cell motility and transcriptional control (27). Originally discovered as an interacting protein of the nuclear thyroid hormone receptor in a yeast two-hybrid screen (13), TRIP6 was later identified as a focal adhesion molecule with the capability to shuttle between cell surface and nucleus (16). TRIP6 is structurally similar to zyxin, LPP (Lipoma Preferred Partner) and Ajuba (27). They possess a proline-rich region and nuclear export signals at their N-termini, and three LIM domains (named by the initials of Lin-11, Isl-1, and Mec-3) at their carboxyl-termini. Through the LIM domain-mediated protein-protein interactions, TRIP6 forms complexes with several molecules involved in actin rearrangement, cell adhesion and migration, at least including p130cas (30), CasL/HEF1 (30), endoglin (24), supervillin (26) and the LPA2 receptor (29). In addition, the most carboxyl-terminal LIM3 and PDZ-binding domain of TRIP6 have been demonstrated to mediate the interaction with the second PDZ domain of human PTPL1/FAP-1 (16) and its mouse homologue, PTP-BL (5). However, the functional significance for this interaction remains to be elucidated.

Lysophosphatidic acid (LPA), a growth factorlike phospholipid, mediates diverse biological responses such as cell migration, cell proliferation and cell survival through the activation of G protein-coupled LPA receptors (15). Among the five membrane-bound LPA receptors (1,3,8,12,18), the LPA1, LPA2 and LPA3 receptors of the EDG
(Endothelial Differentiation Gene) family are structurally similar to each other except for the carboxyl-terminal tails, suggesting that this region may specifically regulate the unique protein-protein interactions and functions of each receptor. Previously we have demonstrated that the carboxyl-terminal tail of the LPA2 receptor, but not LPA1 or LPA3 receptor, interacts with the LIM domains of TRIP6 (29). This association promotes LPA-dependent recruitment of TRIP6 to the focal adhesion sites where it forms complexes with p130cas, FAK, paxillin and c-Src. The function of TRIP6 in cell motility is regulated by c-Src-mediated phosphorylation at Tyr-55 (11). This phosphorylation is required for TRIP6 coupling to the Crk SH2 domain and ERK activation, thereby enhancing LPA-induced morphological changes and chemotaxis.

Cell migration is a dynamic process that requires a tight coordination of various signaling molecules involved in cell adhesion and migration. Several tyrosine kinases and phosphatases have been shown to regulate these signaling events through reversible tyrosine phosphorylation and dephosphorylation of their substrates (28). In particular, the FAK-Src-mediated pathways play a fundamental role in regulating adhesion turnover and disassembly during cell migration (20). To understand how tyrosine phosphorylation and dephosphorylation of TRIP6 modulates its function in cell motility, we explored the possibility whether PTPL1 phosphatase is a candidate responsible for dephosphorylation of TRIP6. Human PTPL1, also known as FAP-1 (Fas-associated Phosphatase 1), PTP1E and PTPN13, and its mouse homologue, PTP-BL, are ~270 kDa cytosolic tyrosine phosphatases which contain an amino-terminal FERM domain, five PDZ motifs and a carboxyl-terminal tyrosine phosphatase catalytic domain (6). The FERM domain functions as membrane-cytoskeleton linkers and may play a role in cytokinesis (9). Several regulators
of actin cytoskeleton have been shown to interact with the PDZ domains of PTPL1, at least including TRIP6, PARG (PTPL1- associated RhoGAP), APC (adenomatous polyposis coli), ephrinB, PRK2 (protein kinase C-related kinase-2), CRIP2 (cysteine-rich intestinal protein 2), and RIL (Reversion-induced LIM), implicating a role for PTPL1 in actin dynamics (6). A number of molecules have been identified as PTPL1 substrates, at least including ephrinB, RIL, IκB and c-Src (4,17,19,23); however, the functional consequences of their dephosphorylation are still poorly understood.

For the first time, we demonstrate that PTPL1 acts as a negative regulator of LPA-induced cell migration. Specifically, PTPL1 dephosphorylates c-Src-phosphorylated TRIP6 in vitro, and inhibits LPA-induced tyrosine phosphorylation of TRIP6 in cells through a direct protein-protein interaction. This dephosphorylation reduces TRIP6 turnover from the sites of adhesions, and inhibits TRIP6 coupling to Crk. As a result, the function of TRIP6 in promoting LPA-induced morphological changes and cell migration is attenuated by PTPL1.

Experimental Procedures

Plasmid Construction

A TRIP6 cDNA fragment encoding the amino acids 1-396 was amplified by polymerase chain reaction (PCR), and inserted into pCMV-Tag2A (Stratagene) to generate the FLAG-TRIP6-Δ(397-476) expression vector. The pEYFP-TRIP6 was constructed by replacing the green fluorescence protein (GFP) cDNA sequences with a PCR product encoding yellow fluorescent protein (YFP) in pEGFP-TRIP6. A PCR product encoding GFP or a cyan fluorescent protein (CFP) was inserted into pCMV5-HA-PTPL1 (10) such
that HA-PTPL1 was tagged inframe with GFP or CFP at its N-terminus, respectively. To express a PTPL1 catalytic domain (designated PTPL1-CD), a cDNA fragment encoding amino acids 2087-2485 of PTPL1 was amplified by PCR from pCMV5-HA-PTPL1, and inserted into pGEX-6P3 (Amersham Biosciences). To inhibit the expression of PTPBL, a mouse homologue of PTPL1, the pSUPER vector was used to direct the expression of a small interfering RNA (siRNA) of PTP-BL (designated pSUPER-si(m)PTPL1), which specifically targets the 21-nt sequences of PTP-BL, 5’-GAGTGAGCATTGCTGACCCTG-3’. A control siRNA (designated siScramble) expression vector was constructed by inserting the 19-nt sequences, 5’-GCGCGCTTTGTAGGATTCG-3’ that do not target any known cellular RNA, into pSUPER vector. The entire sequences of each cDNA clone were verified by automatic DNA sequencing.

In vitro phosphorylation and dephosphorylation assays

*E. coli* BL21(DE3)(LysS) was transformed with pGEX-6P3-TRIP6 or pGEX-6P3-PTPL1-CD. GST-TRIP6 and GST-PTPL1-CD were purified as described previously (29). GST-PTPL1-CD was further digested with PreScission protease (Amersham Biosciences) to cleave GST. One µg of GST-TRIP6 was first phosphorylated by recombinant p60 c-Src (Upstate Biotechnology) as described previously (11). Following reaction, GST-TRIP6-immobilized glutathione beads were washed four times to remove c-Src. These beads were then incubated with 0.1 or 0.5 µg of PTPL1-CD for 10 or 30 min in the phosphatase buffer (25 mM imidazole-HCl, pH 7.2, 1 mg/ml BSA and 1 mM DTT). After SDS-PAGE, the immunoblot was probed with an HRP-conjugated antiphosphoty-
Rosine antibody (PY20H, Santa Cruz Biotechnology) to detect phosphorylated TRIP6. The blot was then stripped and re-probed with an anti-TRIP6 antibody (Bethyl Laboratories).

**Co-immunoprecipitation, immunoblotting and tyrosine phosphorylation of TRIP6**

Transfected HEK 293T cells were starved overnight followed by the incubation with 2 µM LPA for 15 min. Co-immunoprecipitation was performed as described previously (29). Immunoblotting was performed using the antibodies specific to human TRIP6 (Bethyl laboratories), mouse TRIP6 (BD Biosciences), vinculin (Sigma), PTPL1, FLAG epitope, GFP, HA epitope, and β-Actin (Santa Cruz Biotechnology).

To detect tyrosine phosphorylation of the endogenous TRIP6, FLAG-TRIP6 or FLAG-TRIP6-Δ(397-476), transfected SYF+c-Src cells were starved in 0.1% fatty acid-free bovine serum albumin (BSA)-containing Dulbecco’s modified Eagle’s medium (DMEM) for 8h followed by the incubation with 2 µM LPA for 15 min. The endogenous TRIP6 and transfected FLAG-TRIP6 were immunoprecipitated with an anti-TRIP6 mouse antibody (BD Biosciences) and anti-FLAG M2 monoclonal antibody-conjugated agarose beads from the lysates, respectively. After SDSPAGE, tyrosine-phosphorylated TRIP6 was detected by immunoblotting using an HRP-conjugated anti-phosphotyrosine antibody (PY20H, Santa Cruz Biotechnology).

**Time-lapse imaging of live cells**

SYF+c-Src MEFs expressing YFP or CFP fusion proteins of TRIP6 or PTPL1 were plated on glass-bottomed 35-mm tissue culture dishes (MatTek Corp.). Cells were
washed twice with phenol red-free DMEM/F-12 containing 1% fatty acid-free BSA and 10 mM HEPES, pH 7.4, and then incubated with 10 µM LPA. The expression of YFP and CFP fusion proteins was visualized by Olympus IX70 inverted fluorescence microscope using a 100X objective. The YFP images were acquired every 20 sec for 30 min with a Photometrics 1400 charge-coupled device camera under the control of IPLab software (Scanlytics, Inc.).

**Transwell cell migration assays**

Transfected SKOV-3 cells were subjected to a transwell cell migration assay as described previously (11).

**TRIP6 turnover**

SyF+c-Src MEFs expressing YFP-PTPL1 or control YFP were starved for 8h followed by the incubation with 2 µM LPA for 15, 30 and 45 min. Cells were harvested on ice in the Triton-X-containing buffer (1% Triton-X, 10 % glycerol, 150 mM NaCl, 50 mM HEPES, pH 7.4, 1 mM EDTA and 1 mM EGTA) supplemented with a mixture of protease inhibitors and phosphatase inhibitors for 30 min. The detergent-resistant cytoskeletal fractions were collected by centrifugation at 14,000 x g for 15 min. The pellets were dissolved in SDS lysis buffer, boiled, and sonicated to disrupt the cytoskeleton. Immunoblotting was performed to detect TRIP6, β-Actin and vinculin using antibodies specific to these proteins.

**Results and Discussion**
PTPL1 inhibits LPA-induced tyrosine phosphorylation of TRIP6 through a direct interaction with TRIP6.

Previously it has been shown that the carboxyl-terminal LIM3 and PDZ-binding domain of TRIP6 interacts with the second PDZ domain of human PTPL1 and its mouse homologue, PTP-BL, in the yeast two-hybrid systems (5,16). However, the functional significance for this interaction has not yet been elucidated. In search of the candidate phosphatase of TRIP6, we tested the ability of PTPL1 to dephosphorylate phosphotyrosine-55 of TRIP6 in vitro. We first phosphorylated GST-TRIP6 with recombinant c-Src kinase. Following the reaction, c-Src was removed by extensive washing, and GST-TRIP6 was incubated with the catalytic domain of PTPL1 for 15 or 30 min. The result showed that the c-Src-phosphorylated TRIP6 was dephosphorylated in the presence of PTPL1 in a dose- and time-dependent manner (Fig. 1A). To investigate if PTPL1 regulates TRIP6 phosphorylation in cells, GFP-PTPL1 or control GFP was overexpressed in Src\textsuperscript{−/−}, Yes\textsuperscript{−/−}, Fyn\textsuperscript{−/−} triple knockout mouse embryonic fibroblasts (MEFs) in which c-Src has been reconstituted (designated SYF+c-Src MEFs), and tyrosine phosphorylation of TRIP6 was determined after LPA stimulation for 15 min. As shown in Fig. 1B, LPA-induced tyrosine phosphorylation of endogenous TRIP6 was abolished by overexpression of PTPL1. In contrast, inhibiting the expression of PTP-BL (a mouse homologue of PTPL1) by a specific siRNA (designated si(m)PTPL1) enhanced LPA-induced tyrosine phosphorylation of TRIP6 in SYF+c-Src MEFs (Fig. 1C), suggesting a physiological relevance of this regulation.

Previously it has been reported that PTP-BL, a mouse homologue of PTPL1, dephosphorylates phosphotyrosine-416 of c-Src, thereby preventing autophosphoryla-
tion of c-Src and inactivating its kinase activity (19,23). Although PTPL1 may mediate TRIP6 dephosphorylation through a direct interaction with TRIP6, it is also possible that PTPL1 indirectly inhibits tyrosine phosphorylation of TRIP6 by attenuating c-Src kinase activity. To rule out this possibility, we compared tyrosine phosphorylation of wild-type TRIP6 and a TRIP6-∆(397-476) deletion mutant that lacks LIM3 and PDZ-binding domains required for PTPL1 interaction. The association of PTPL1 with TRIP6 but not TRIP6-∆(397-476) mutant was first confirmed in HEK 293T cells where LPA stimulation for 15 min promoted the association of PTPL1 with TRIP6, whereas deletion of the LIM3 and PDZ-binding domain of TRIP6 abolished this interaction (Fig. 2A). Importantly, PTPL1 attenuated c-Src-mediated tyrosine phosphorylation of TRIP6, but not TRIP6-∆(397-476) mutant (compare lanes 3, 4 with lanes 1, 2; and compare lanes 7, 8 with lanes 5, 6, Fig. 2B). This result suggests that PTPL1 mediates TRIP6 dephosphorylation through a direct protein-protein interaction.

PTPL1 reduces TRIP6 turnover at the sites of adhesions, and inhibits LPA-induced morphological changes when co-expressing with TRIP6.

Previously we have shown that in serum-free conditions, TRIP6 is diffusely present in the cytosol. Upon stimulation with LPA for 15-20 min, TRIP6 is targeted to focal adhesions and colocalized with actin cytoskeleton (29). c-Src-mediated phosphorylation of TRIP6 at Tyr-55 does not affect these signaling events, but is important for TRIP6 turnover at the sites of adhesions (11). Accordingly, PTPL1-dependent dephosphorylation may regulate TRIP6 turnover at focal adhesions. To examine the turnover of cytoskeleton-associated TRIP6 following LPA stimulation, we set up the experiment to de-
termine LPA-induced accumulation of TRIP6 in the detergent-resistant cytoskeletal fractions. Therefore, SYF+c-Src cells expressing YFP-PTPL1 or control YFP were starved for 8h followed by the stimulation with LPA for 15, 30 or 45 min, and the Triton-X-insoluble fractions were collected. Our results showed that LPA stimulation rapidly induced the accumulation of TRIP6 in the insoluble cytoskeletal fractions (Fig. 3A). It reached the highest level after 15-min treatment. Subsequently, TRIP6 was dissociated from cytoskeleton; however, the turnover of cytoskeleton-associated TRIP6 was significantly delayed in cells overexpressing PTPL1 (Fig. 3B). Compared to TRIP6, significant amounts of vinculin were already present in the insoluble cytoskeletal fractions. In the control SYF+c-Src cells, LPA stimulation slightly increased the accumulation of vinculin in the detergent-resistant fractions, but the turnover of vinculin was much slower than TRIP6 (Fig. 3A). In the cells overexpressing PTPL1, LPA stimulation did not further increase the accumulation or induce the turnover of vinculin in detergent-resistant cytoskeletal fractions. These results suggest that LPA induces a rapid assembly and disassembly of focal adhesion molecules in SYF+c-Src cells; however, this dynamic process is retarded by PTPL1 overexpression.

LPA stimulation rapidly induces cytoskeletal rearrangement with cellular morphological changes through the coordinate activation of Rho GTPases (21). Previously we have demonstrated that overexpression of TRIP6, but not TRIP6-Y55F mutant, promoted LPA-induced morphological changes in SYF+c-Src MEFs. However, overexpression of TRIP6 in Src-null SYF MEFs not only did not promote LPA-induced morphological changes but even retards this dynamic process (11). One explanation is that TRIP6 forms stable complexes with other signaling molecules at the sites of adhesions,
and the accumulated mature adhesions perturb LPA-induced adhesion disassembly. Analogously, PTPL1-dependent dephosphorylation of TRIP6 may negatively regulate LPA-induced morphological changes. To explore this possibility, time-lapse imaging of live cells was performed in SYF+c-Src cells expressing YFP or YFP-TRIP6 with either CFP or CFP-PTPL1. Cells were washed twice with 1% fatty acid-free BSA-containing medium, and the expression of these proteins was verified by fluorescence microscopy. Subsequently, LPA was added to the cells and the YFP images of live cells were acquired by time-lapse fluorescence microscopy. Our results showed that the cells expressing control YFP and CFP started to retract their cell bodies soon after LPA stimulation for 5 min, and almost completely rounded up after 15 min of treatment (Fig. 3C, 1st panel, and Supplemental video 1, YFP+CFP.mov). However, this process became much slower when CFP-PTPL1 was coexpressed (Fig. 3C, 2nd panel, and Supplemental video 2, CFP+PTPL1.mov). Overexpression of YFP-TRIP6 accelerated the rate of LPA-induced morphological changes, and cells started to contract soon after LPA treatment for one min (Fig. 3C, 3rd panel, and Supplemental video 3, YFP-TRIP6+CFP.mov). In contrast, in cells coexpressing YFP-TRIP6 and CFP-PTPL1, LPA-induced morphological changes were almost completely blocked (Fig. 3C, 4th panel, and Supplemental video 4, YFP-TRIP6+CFP-PTPL1.mov). It is apparent that the number of focal adhesions and the accumulation of YFP-TRIP6 at the sites of adhesions were increased when CFP-PTP-L1 was co-expressed. These results suggest that the relative expression of phosphorylated and dephosphorylated TRIP6 is critical for TRIP6 function in regulating LPA-induced morphological changes.
**PTPL1 reduces LPA-induced association of TRIP6 with Crk, and attenuates TRIP6 function in LPA-induced cell migration.**

Previously we have demonstrated that the pY55QAP motif of human TRIP6 or pY55QPP motif of mouse TRIP6 creates a docking site for the Crk SH2 domain once Tyr-55 is phosphorylated by c-Src (11). Since PTPL1 mediates dephosphorylation of phosphotyrosine-55 of TRIP6, it would be expected to negatively regulate this association. Indeed, our result showed that LPA-induced interaction of TRIP6 with CrkI was significantly reduced by PTPL1 (Fig. 4A). This result implies a role for PTPL1 in regulating TRIP6 function in LPA-induced cell migration.

So next we expressed PTPL1 with TRIP6 in SKOV-3 ovarian cancer cells in which c-Src is highly expressed, and examined LPA-induced cell migration. Our result showed that overexpression of PTPL1 reduced LPA-induced transwell cell migration (Fig. 4B). In the absence of ligand stimulation, the relative cell migration rate was slightly higher in cells overexpressing TRIP6. However, the ability of TRIP6 to promote LPA-induced cell migration was significantly attenuated by PTPL1 overexpression.

In summary, TRIP6 function in LPA-induced adhesion turnover and cell migration is coordinately regulated through c-Src and PTPL1-dependent reversible tyrosine phosphorylation and dephosphorylation. Another example for this regulation is ephrinB. It has been reported that Src family kinases mediate ephrinB phosphorylation upon the engagement of ephrinB with Eph receptor, and regulate ephrinB-stimulated angiogenic sprouting in primary endothelial cells. With delayed kinetics, ephrinB recruits PTP-BL and is dephosphorylated, which switches the Src-mediated signaling to PDZ-dependent signaling (19).
Thus far, several tyrosine phosphatases have been shown to modulate cell motility, at least including PTP-PEST, PTP-α, PTP-1B, SHP-2 and low molecular weight PTP (LMW-PTP) (2,14,22,31,32). Overexpression or inhibition of these tyrosine phosphatases may perturb adhesion turnover and impair cell motility. For example, overexpression of PTP-PEST, a phosphatase for tyrosine-phosphorylated p130cas, leads to the inactivation of Rac1 and defective cell migration (7,25). Intriguingly, the PTP-PEST-/- MEFs also show impaired cell spreading and migration (2). These findings suggest that an appropriate regulation of the tyrosine phosphatase activity of PTP-PEST is important for efficient cell migration. Likewise, overexpression of PTPL1 slows TRIP6 turnover at the sites of adhesions and negatively regulates TRIP6 function to promote LPA-induced cell migration. Since PTPL1 associates with a number of actin-associated molecules, it will be an important task to determine if PTPL1 plays a general role in modulating cell motility through dephosphorylation of other substrates involved in cell adhesion and migration.

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References


FIG. 1 PTPL1 mediates dephosphorylation of phosphotyrosine-55 of TRIP6 in vitro, and inhibits LPA-induced tyrosine phosphorylation of TRIP6 in cells. A. PTPL1 dephosphorylates c-Src-phosphorylated TRIP6 in a dose- and time-dependent manner in vitro. One µg of purified recombinant GST-TRIP6 was phosphorylated by c-Src in vitro. After extensive washing to remove c-Src, phosphorylated GST-TRIP6 was incubated with 0.1 or 0.5 µg of the catalytic domain of PTPL1 (PTPL1-CD) for 30 min (upper panel), or with 0.5 µg of PTPL1-CD for 10 or 30 min (bottom panel). After SDS-PAGE, the im-
munoblot was probed with an anti-phosphotyrosine antibody (PY20H) to detect phosphorylated TRIP6. The immunoblot was then stripped and reprobed with a TRIP6-specific antibody. B. PTPL1 overexpression eliminates LPA-induced tyrosine phosphorylation of TRIP6 in SYF+c- Src cells. SYF+c-Src cells were transiently transfected with pEGFP or pEGFP-PTPL1. Cells were starved in 0.1% fatty acid-free BSA-containing medium for 8h followed by the incubation with 2 µM LPA for 15 min. The endogenous TRIP6 was immunoprecipitated with a TRIP6-specific monoclonal antibody or a control mouse IgG. After SDS-PAGE, the immunoblot was probed with an antiphosphotyrosine antibody to detect tyrosine phosphorylated TRIP6. The bottom three panels are immunoblots showing the expression of TRIP6, GFP-PTPL1 and GFP in the whole cell lysates, respectively. C. Inhibition of PTPL1 expression enhances LPA-induced tyrosine phosphorylation of TRIP6 in SYF+c-Src cells. SYF+c-Src cells were transiently transfected with a control pSUPER-siScramble vector expressing a control siRNA or pSUPER-si(m)PTPL1 vector expressing an siRNA of PTP-BL, a mouse homologue of PTPL1. LPA-induced tyrosine phosphorylation of TRIP6 was determined as described in B. The bottom two panels show the expression of endogenous PTPL1 and TRIP6 in the whole cell lysates, respectively.
FIG. 2 Deletion of the carboxyl-terminal LIM3 and PDZ-binding domains of TRIP6 disrupts its interaction with PTPL1, and abolishes PTPL1-mediated dephosphorylation of TRIP6. A. A TRIP6-Δ(397-476) mutant that lacks the LIM3 and PDZ-binding domains cannot bind to PTPL1. HEK 293T cells transiently expressing FLAG-TRIP6 or FLAG-TRIP6-Δ(397-476) were starved overnight and then stimulated with LPA for 15 min. TRIP6 or TRIP6-Δ(397-476) was immunoprecipitated with an anti-FLAG M2 monoclonal antibody. After SDS-PAGE, the immunoblot was probed with an anti-PTPL1 antibody. The blot was then stripped and reprobed with an anti-FLAG antibody to detect the immunoprecipitated TRIP6 or TRIP6-Δ(397-476). The bottom panel shows the expression of endogenous PTPL1 in the whole cell lysates. B. PTPL1 overexpression does not affect tyrosine phosphorylation of TRIP6-Δ(397-476). FLAG-TRIP6 or FLAG-TRIP6-Δ(397-476) was expressed in HEK 293T with HA-c-Src, GFP or GFP-PTPL1 as indicated. After starvation overnight, cells were stimulated with LPA for 15 min. The levels of tyrosinephosphorylated TRIP6 or TRIP6-Δ(397-476) were determined as described in A. The bottom three panels show the expression of GFP-PTPL1, GFP and HA-c-Src in the whole cell lysates, respectively.
FIG. 3 PTPL1 reduces the turnover of cytoskeleton-associated TRIP6, and inhibits LPA-induced morphological changes. A and B. LPA induces a rapid accumulation and turn-
over of TRIP6 in the detergent-resistant cytoskeletal fractions, whereas PTPL1 slows this dynamic process. A. SYF+c-Src fibroblasts transiently expressing YFP or YFP-PTPL1 were starved for 8h followed by the stimulation with LPA for 15, 30 or 45 min, and then harvested in 1% Triton X-100-containing buffer on ice for 30 min. The insoluble cytoskeletal fractions were collected by centrifugation at 14,000 x g, and dissolved in SDS lysis buffer. After boiling and sonication to disrupt cytoskeleton, equal amounts of protein were subjected to immunoblotting using the antibodies specific to TRIP6, β-Actin and vinculin, respectively. The right panel shows the expression of YFP-PTPL1 in the whole cell lysates. B. The expression levels of TRIP6 in the insoluble cytoskeletal fractions were quantified using NIH IMAGE J software program and were compared with that in the absence of LPA. The result shown is a representative from three independent experiments. C. The ability of TRIP6 to promote LPA-induced morphological changes is inhibited by PTPL1. SYF+c-Src cells transiently expressing YFP or YFP-TRIP6 with CFP or CFP-PTPL1 were washed twice with phenol red-free DMEM/F-12 containing 1% fatty acid-free BSA, and then stimulated with 10 µM LPA for 20 min. The YFP images of live cells were acquired very 20 sec for 20 min by inverted fluorescence microscope using a 100X objective under the control of IPLab software. Results shown are the images captured at various times, and are representative of five independent experiments.
FIG. 4 PTPL1 negatively regulates TRIP6 function in Crk coupling and LPA-induced cell migration. A. PTPL1 attenuates LPA-induced association of TRIP6 with CrkI. HEK 293T cells expressing FLAG-TRIP6, GFP-CrkI, HA-c-Src without or with HA-PTPL1 were starved overnight and then stimulated with LPA for 15 min. TRIP6 was immunoprecipitated with an anti-FLAG M2 monoclonal antibody-conjugated agarose beads. After SDS-PAGE, the immunoblot was probed with an anti-GFP antibody to detect co-immunoprecipitated CrkI. The bottom four panels show the expression of GFP-CrkI, HA-c-Src, FLAG-TRIP6 and total PTPL1 in the whole cell lysates, respectively. B. PTPL1 attenuates the ability of TRIP6 to promote LPA-induced cell migration. SKOV-3 cells were transiently transfected with pEGFP or pEGFP-TRIP6 without or with pCMV5-HA-PTPL1. Cells were washed with 1% fatty acid-free BSA-containing medium and then subjected to a transwell cell migration assay. LPA was added to the bottom chamber of the transwell, and cells were allowed to migrate for 6h. Cells migrated to the fibronectin-coated bottom filter were fixed, and the GFP-positive cells were counted under fluorescence microscope. The migration rate was determined as the relative rate of migrated
cells compared to the migrated GFP-expressing cells in the absence of LPA, and was normalized by transfection efficiency. The results shown are the mean±S.E. of three independent experiments. *p<0.01 versus LPA-stimulated GFP-expressing cells. **p<0.05 versus LPA-stimulated GFP-TRIP6-expressing cells (Student’s t test).
CHAPTER 5

CONCLUSION AND DISCUSSION

Summary

In this study, we identify TRIP6 as an LPA₂ interacting protein. The function of TRIP6 in mediating LPA-induced cell migration is regulated by c-Src-dependent tyrosine phosphorylation and PTPL₁ phosphatase-dependent dephosphorylation. TRIP6 binds to the carboxyl-terminal tail of LPA₂, but not LPA₁ or LPA₃, through its LIM2 and LIM3 domains in an LPA-dependent manner. TRIP6 associates with several focal adhesion molecules such as FAK, paxillin, p130_Cas and c-Src, and co-localizes with actin cytoskeleton. Overexpression of TRIP6 increases LPA-induced cell migration, whereas knocking down TRIP6 with a TRIP6-specific siRNA blocks it in SKOV3 cells.

TRIP6 is tyrosine phosphorylated by c-Src primarily at the tyrosine 55 residue after LPA stimulation, which serves a mechanism how TRIP6 regulates LPA-induced cell migration. Phosphorylation of TRIP6 at Tyr-55 residue converts the pY₅₅QAP motif into a docking site of the SH2 domain of Crk. It has been shown that Crk is important for cell motility because it couples to DOCK180 Rac GEF and activates Rac. Moreover, phosphorylation of TRIP6 by c-Src is required for LPA-induced ERK activation and cellular morphological changes. Activated ERKs have been implicated in the regulation of focal adhesion turnover and cell motility. In SYF cells, where Src, Fyn and Yes genes of the Src kinase family have been disrupted, LPA-induced ERK activation is much lower compared to that SYF cells reconstituted with c-Src (SYF+c-Src). Knockdown of
TRIP6 with its specific siRNA decreases LPA-induced ERK activation in SYF+c-Src cells, whereas overexpressing TRIP6, but not the TRIP6-Y55F mutant, increases it. Similarly, LPA-induced morphological changes are faster in SYF+c-Src cells overexpressing TRIP6, but not the TRIP6-Y55F mutant. In SKOV3 cells, Y55F mutation also reduces the ability of TRIP6 to promote LPA-induced cell migration. We conclude that LPA-stimulated c-Src phosphorylation of TRIP6 is required for its function in mediating Crk coupling and ERK activation. As a result, it promotes cellular morphological changes and cell migration.

If the function of TRIP6 in cell migration is promoted by c-Src kinase, it would be expected that its function could be negatively regulated by a tyrosine phosphatase. Since PTPL1 phosphatase has been shown to interact with the carboxyl-terminal PDZ binding domain of TRIP6, we tested the possibility whether PTPL1 regulates TRIP6 function. In this report, we confirm the interaction of TRIP6 and PTPL1 in cells, and provide evidence that PTPL1 dephosphorylates TRIP6 after LPA stimulation for 15 minutes. Together the results show that LPA-stimulated phosphorylation of TRIP6 reaches the highest levels at 15 minutes of LPA treatment and then returns to baseline after 30 minutes in SYF+c-Src cells. PTPL1 dephosphorylates TRIP6 right after LPA stimulation for 15 minutes. PTPL1 directly dephosphorylates TRIP6 since PTPL1 only reduces the phosphorylation levels of TRIP6 but not the TRIP6-ΔLIM3+PDZ mutant that cannot interact with PTPL1. The ability of TRIP6 to bind Crk is greatly attenuated in cells overexpressing PTPL1. Dephosphorylation of TRIP6 by PTPL1 results in the accumulation of TRIP6 in the fractions of detergent-resistant actin cytoskeleton. Consequently, overexpression of PTPL1 reduces TRIP6 function in promoting LPA-induced cellular morpho-
logical changes and cell migration. Taken together, LPA-induced cell migration is positively regulated by c-Src-dependent phosphorylation of TRIP6 and negatively regulated by PTPL1-dependent dephosphorylation of TRIP6.

Discussion

TRIP6 and LPA Signaling

Although TRIP6 specifically binds to the LPA₂ receptor and regulates LPA signaling, TRIP6 may also indirectly regulate other LPA receptors or cellular receptors through the heterodimerization of the LPA receptors or cross-talk among different cellular receptors. In addition to the LPA receptors, for example, TRIP6 has been shown to interact with other receptors such as TNF, IL-1, TLR2 and Nod1[44], indicating its diverse role in cellular signaling events. On the other hand, however, TRIP6 may involve in different extracellular signaling pathways to the same cellular response.

In addition to the regulation of cell motility and gene transactivation, a focal adhesion molecule like TRIP6 may also play a role in cell cycle progression or cell survival. TRIP6 has been shown to activate NF-κB[44], implicating a potential role of TRIP6 in cell survival. Furthermore, PTPL1, also known as Fas-associated protein 1 (FAP-1), has been shown to regulate anti-Fas-induced apoptosis[64]. Likewise, LPA protects ovarian cancer cells from Fas-mediated apoptosis[77]. Whether the association of PTPL1 with TRIP6 plays a role in LPA-mediated anti-apoptotic signaling remains to be investigated. In conclusion, TRIP6 may play multifunctional roles in different signaling events, at least including cell migration, cell proliferation and cell survival.
TRIP6 and Zyxin Family

Zyxin family proteins not only share the homology in protein structure, but also show similarity in cellular localization and functions in cell migration, nucleocytoplasmic shuttling and transactivation[53]. So far, the knock-out mice of zyxin family members (zyxin, Ajuba and LIMD1) show no significant effects in development and survival[78-80]. It may be due to the functional overlapping among the family members, implicating the functional redundancy of zyxin family members. Zyxin, same as TRIP6, has been shown to interact with p130Cas and CasL/HEF1, and regulates cell migration[58]. However, zyxin cannot be phosphorylated by c-Src (Lai and Lin, unpublished), indicating that the mechanism that regulates zyxin function is different from TRIP6. Ajuba also interacts with p130Cas. However, different from zyxin and TRIP6 binding, which is mediated by their LIM region, this binding is through it N-terminal proline region[79]. Ajuba-/- MEFs show abnormal lamellipodia production and reduced cell migration by wound healing assay[79]. In addition, Ajuba binds to Grb2 to regulate Ras-dependent ERK activation[50]. The function of LPP is also important for cell migration. It expresses at high levels in vascular smooth muscle cells and overexpression of LPP increase EGF-stimulated migration of vascular smooth muscle cells[81]. Rho kinase inhibitor Y-27632 treatment can disrupt the focal adhesion localization of LPP[81]. Depletion of FAK expression (FAK-/- fibroblasts) also decreases LPP expression, and restoring LPP expression in these cells enhanced cell spreading[82]. Moreover, LPP and TRIP6, but nor zyxin, have been shown to interact with supervillin, a myosin II and F-actin binding protein, which negatively regulates focal adhesion turnover, stress fiber formation and cell-
substrate adhesion[75]. It is speculated that TRIP6/LPP may compete with myosin and F-actin to bind to supervillin, and rescue supervillin effects on cell motility.

In addition to the regulation of cell motility, zyxin family proteins show similar properties in shuttling between the cytosol and nucleus, and have been implicated in bringing extracellular signals into nucleus[53]. Ajuba and LIMD1 have been shown to activate NF-κB and AP-1 through the binding to p62 and TRAF6[80, 83], while TRIP6 activates NF-κB through the interaction with TNF and IL-1 receptors, p65 and TRAF2[44]. Although there is no direct evidence showing that LPP is involved in gene transactivation, it was first identified in a chromosome translocation event in lipoma, in which the C-terminal LIM domains of LPP is fused to the N-terminal region of the high mobility group protein HGMIC, and generated a nuclear-localized protein which is deregulated and may be involved in the tumorigenesis of lipoma[49]. It has been speculated that the LIM domains of LPP may be responsible for the nuclear translocation of the HMGIC/LPP fusion protein.

Although most of the zyxin family members share similar roles in the regulation of cell migration and transcriptional activity, it is clear that the mechanisms of their regulation are different. The specificity and mechanisms by which these zyxin family members regulate cell motility remains to be investigated.

Future Studies

Although our current studies firmly establish the fact that LPA-induced cell migration is positively regulated by c-Sr-dependent phosphorylation of TRIP6 and negatively regulated by PTPL1-dependent dephosphorylation of TRIP6, TRIP6 and other
zyxin family proteins interact with many other cellular molecules involved in different cellular functions which remain to be investigated. Many physiological and pathophysiological aspects of TRIP6 and LPA also remain to be explored in the future. Here are few examples.

1. To further examine the specificity of TRIP6 in LPA$_2$-mediated signaling pathway and the possible roles of TRIP6 in regulation of LPA function other than cell migration, such as mitogenesis, differentiation, cell survival, angiogenesis and inflammation.

2. To demonstrate more physiological functions of TRIP6 in cells other than LPA signaling.

3. To investigate the role of TRIP6 in nuclear localization and signal transduction.

4. To examine the effects of TRIP6 in cancer cell progression and metastasis.


83. Feng, Y. and G.D. Longmore, *The LIM protein Ajuba influences interleukin-1-induced NF-kappaB activation by affecting the assembly and activity of the pro-