Published in final edited form as:
J Biol Chem. 2007 December 28; 282(52): 37759–37769. doi:10.1074/jbc.M705025200.

The Lysophosphatidic Acid 2 Receptor Mediates Down-regulation of Siva-1 to Promote Cell Survival*,χ

Fang-Tsyr Lin[‡,1], **Yun-Ju Lai**[‡,2], **Natalia Makarova**[§], **Gabor Tigyi**[§], and **Weei-Chin Lin**[‡,§]

[‡]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294

[¶]Division of Hematology and Oncology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

[§]Department of Physiology, University of Tennessee Health Sciences Center, Memphis, Tennessee 38163

Abstract

Lysophosphatidic acid (LPA) promotes cell survival through the activation of G protein-coupled LPA receptors. However, whether different LPA receptors activate distinct anti-apoptotic signaling pathways is not yet clear. Here we report a novel mechanism by which the LPA_2 receptor targets the proapoptotic Siva-1 protein for LPA-dependent degradation, thereby attenuating Siva-1 function in DNA damage response. The carboxyl-terminal tail of the LPA_2 receptor, but not LPA_1 or LPA_3 receptor, specifically associates with the carboxyl cysteine-rich domain of Siva-1. Prolonged LPA stimulation promotes the association of Siva-1 with the LPA_2 receptor and targets both proteins for ubiquitination and degradation. As a result, adriamycin-induced Siva-1 protein stabilization is attenuated by LPA in an LPA_2-dependent manner, and the function of Siva-1 in promoting DNA damage-induced apoptosis is inhibited by LPA pretreatment. Consistent with this result, inhibition of the LPA_2 receptor expression increases Siva-1 protein levels and augments adriamycin-induced caspase-3 cleavage and apoptosis. Together, these findings reveal a critical and specific role for the LPA_2 receptor through which LPA directly inactivates a critical component of the death machinery to promote cell survival.

Lysophosphatidic acid (LPA)[3] is a growth factor-like phospholipid that mediates diverse biological events such as mitogenesis, differentiation, cell survival, platelet aggregation, inflammation, angiogenesis, and cell migration (1). At least five membrane-bound G protein-coupled LPA receptors have been identified. The LPA_1, LPA_2, and LPA_3 receptors belong to the endothelial differentiation gene family, whereas the LPA_4 and LPA_5 receptors are structurally distinct from the others (2–6). Substantial evidence has shown that LPA can protect cells from serum deprivation-induced apoptosis, prevent chemotherapeutic agents-induced apoptosis, and block death receptor-mediated apoptosis in a variety of cells,

*This work was supported by National Institutes of Health Grant CA100848 (to F.-T. L). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

χThe on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[1]To whom correspondence should be addressed: Dept. of Cell Biology, The University of Alabama at Birmingham, MCLM 360A, 1918 University Blvd., Birmingham, AL 35294-0005. Tel.: 205-975-5060; Fax: 205-975-5648; flin@uab.edu. .

[2]Recipient of an American Heart Association predoctoral fellowship.

[3]The abbreviations used are: LPA, lysophosphatidic acid; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione *S*-transferase; siRNA, small interfering RNA; aa, amino acid(s); MEF, mouse embryonic fibroblast; HA, hemagglutinin; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

suggesting that LPA is a survival factor (7–9). Several LPA-induced signaling pathways have been defined for these protective effects (8). For example, the activation of AKT/PKB by LPA stimulation leads to the phosphorylation and inactivation of BAD and procaspase-9, thereby suppressing the apoptotic signaling pathways (10, 11). In addition, LPA-induced activation of ERKs has been reported to protect fibroblasts from serum deprivation-induced apoptosis (7). LPA also induces gene expression of NF-κB, which activates the transcription of several anti-apoptotic proteins to promote cell survival (12). As the levels of LPA are increased in ovarian cancer cells, LPA may contribute to the resistance to apoptosis and tumor progression (13). Thus far, the specificity and mechanisms by which different LPA receptors regulate these signaling events are not yet fully understood.

The carboxyl-terminal tails of the LPA_1, LPA_2, and LPA_3 receptors are structurally distinct form each other, suggesting that this region may specifically regulate the unique protein-protein interactions and functions of each receptor. Therefore, we used the carboxyl-terminal tail of the LPA_2 receptor as bait in a yeast two-hybrid screen to identify molecules specifically involved in the function of the LPA_2 receptor. In addition to TRIP6, a focal adhesion molecule that regulates LPA-induced cell migration (14), the proapoptotic Siva-1 protein was found to interact with the LPA_2 receptor with a high affinity.

Siva-1 is a proapoptotic protein originally identified as a CD27-binding protein (15). The structure of Siva-1 protein contains a death domain homology region in the internal sequences and two zinc finger-like cysteine-rich domains in the carboxyl terminus; however, they lack histidine residues (see Fig. 1*D*) (15). Several lines of evidence suggest that Siva-1 is a proapoptotic protein. For example, Siva-1 gene expression is activated during DNA damage response and stroke injury (16–19). Overexpression of a metastasis suppressor, TIP30, activates Siva-1 transcription in small cell lung cancers (19), and the transcription factors, including p53 and E2F1, directly bind to the Siva-1 promoter and transcriptionally activate Siva-1 expression during DNA damage response (17). Siva-1 is essential for p53-dependent neuronal cell death (20). In addition to inducing apoptosis of T lymphocytes through a caspase-dependent mitochondrial pathway (21), Siva-1 may negatively regulate NF-κB activity in T cell receptor-mediated activation-induced cell death (22). Siva-1 also binds to Bcl-xL through its death domain homology region and inhibits Bcl-xL-mediated protection against UV irradiation-induced apoptosis (23). Previously it has been reported that Siva-2, a minor form of alternative splice variant that lacks exon 2-coding sequences and most of the death domain homology region, is less apoptotic (24). However, a recent report shows that overexpression of Siva-2 similarly induces apoptosis in T lymphocytes (21). Although it has been shown that phosphorylation of Siva-1 by ARG (c-Abl-related gene) kinase at Tyr-34 is required for ARG-mediated apoptosis during oxidative stress (25), the mechanisms that regulate Siva-1 function in apoptosis are still very elusive.

In this report, we characterize the functional significance of the interaction between Siva-1 and the LPA_2 receptor. Our results show that this association promotes LPA-dependent ubiquitination and degradation of Siva-1 protein, thereby down-regulating the proapoptotic activity of Siva-1 during DNA damage response. Thus, in addition to activating AKT/PKB and ERK pathways to promote cell survival, LPA directly inhibits the proapoptotic function of Siva-1 through specific binding of the LPA_2 receptor to Siva-1.

EXPERIMENTAL PROCEDURES

Plasmid Construction

To construct the Siva-1 expression vector, a human Siva-1 cDNA isolated from a yeast two-hybrid screen, which contains six base pairs 5′ to the translation start sites, the entire Siva-1 coding sequences and part of the 3′-un-translated region, was cloned into pcDNA3 for the

expression of full-length Siva-1. This cDNA fragment was also inserted into pCMV-Tag2A, pCMV-Tag3A (Stratagene), pEGFP-C1, pHcRed1-C1 (Clontech), pGEX-6P-3 (Amersham Biosciences), or pcDNA3-HA expression vector, respectively, such that these proteins were tagged in-frame with a FLAG epitope, a Myc epitope, a green fluorescent protein (GFP), an HcRed1 fluorescence protein, a glutathione *S*-transferase (GST), or a hemagglutinin epitope at their amino termini. Different cDNA clones of Siva-1 deletion mutants were constructed by site-directed mutagenesis (Promega) and subcloned into pEGFP-C1 and pCMV-Tag2A. The entire sequences of each cDNA clone were verified by automatic DNA sequencing. The pSUPERIOR.puro vector (OligoEngine) was used to direct the expression of a GFP siRNA (26), a scrambled siRNA, a mouse LPA_2 siRNA, or a mouse Siva-1 siRNA that targets the 19-nucleotide sequences, 5′-GCGCGCTTTGTAGGATTCG-3′ (siScramble), 5′-CGGCACCGCAGTGTGATGG-3′ (siLPA_2), or 5′-GCAGCTCCTTTTCCAAGGG (siSiva-1).

Yeast Two-hybrid Screening

The pAS-LPA_2-CT (aa 296–351) (14) was used as bait to screen a HeLa cell cDNA library (cDNA constructed in pGAD GH) as described previously (14).

Cellular Co-immunoprecipitation and Immunoblotting

Cellular co-immunoprecipitation of the LPA receptors with the interacting proteins was performed as described before (14).

To detect cellular interaction of Siva-1 and the LPA_2 receptor at physiological levels, mouse embryonic fibroblasts (MEFs) deficient in LPA_1 and LPA_2 were isolated from $lpa_1^{-/-}$, $lpa_2^{-/-}$ double knock-out mice (27) and immortalized with SV 40 large T antigen. These cells were stably transfected with a puromycin resistance gene expression vector and either pCMV-FLAG empty vector (Stratagene) (designated DKO-mock) or pCMV-FLAG-LPA_2 (designated DKO-LPA_2). Semi-quantitative RT-PCR analysis was performed to select cell lines expressing FLAG-LPA_2 mRNA at the levels comparable with the endogenous LPA_2 in wild-type MEFs. Five mg of total lysates were isolated from DKO-mock and DKO-LPA_2 MEFs treated with 20 μ_M MG-132 or not for 2 h, and the LPA_2 receptor was immunoprecipitated with anti-FLAG M2 monoclonal antibody-conjugated agarose beads (Sigma). The co-immunoprecipitated endogenous Siva-1 was detected with an affinity-purified anti-Siva-1 polyclonal antibody raised against human Siva-1 peptide (aa 30–44). The blot was then reprobed with an anti-human LPA_2 receptor polyclonal antibody (28).

To detect ubiquitinylated Siva-1 and the LPA_2 receptor, HA-ubiquitin was expressed with FLAG-Siva-1 or FLAG-LPA_2 receptor in HEK 293T cells. The cells were starved overnight and then pretreated with 20 μ_M MG-132 (Calbiochem) or vehicle (Me_2SO) for 1 h. Subsequently, the cells were incubated with LPA for 60 min and harvested in 1× radioimmune precipitation assay buffer. FLAG-tagged Siva-1 or LPA_2 receptor was immunoprecipitated with anti-FLAG M2 monoclonal antibody-conjugated agarose beads (Sigma), and the immunoblot was probed with an anti-HA polyclonal antibody (Santa Cruz Biotechnology) to detect ubiquitinylated proteins.

To determine whether Siva-1 is expressed in the nucleus, NIH 3T3 cells transiently expressing Siav-1 without or with FLAG-LPA_2 were starved in 0.1% fatty acid BSA-containing medium overnight followed by the incubation with 2 μ_M LPA for 1 h and then harvested for subcellular fractionation in hypotonic solution (20 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1% Nonidet P-40). The enriched nuclei were dissolved in 1% SDS lysis buffer. 1% SDS was also added to the supernatant, which includes cytosol, subcellular organelles, and plasma membrane. After sonication, the total

cell lysates, nuclear extract, and supernatant were subjected to immunoblotting using an anti-Siva-1 antibody. The blot was then probed with an anti-vinculin antibody (BD Biosciences) and an anti-histone antibody (Roche Applied Science) as a cytosolic and nuclear marker, respectively.

To study the effect of LPA on the turnover of Siva-1 and the LPA_2 receptor, NIH 3T3 cells were transfected with vectors expressing FLAG-Siva-1 or FLAG-LPA_2 receptor by electroporation (Gene Pulser; Bio-Rad). The cells were starved for 2 h, pretreated with 20 μg/ml cycloheximide for 5 min, and then incubated with LPA for various times. FLAG-Siva-1 in the whole cell lysates and the immunoprecipitated FLAG-LPA_2 receptor were detected by immunoblotting using an anti-FLAG polyclonal antibody (Sigma). The immunoblots were probed with either an anti-GAPDH antibody (Alexis) or an anti-β-actin antibody (Sigma) as a loading control. Similar procedure was performed to determine the turnover rate of FLAG-Siva-1 in NIH 3T3 cells after treatment with 10 μ_M LPA and 10 μ_M adriamycin for 20 h.

The RH7777 rat hepatoma cell line was purchased from the American Type Culture Collection and stably transfected with pCMV-FLAG empty vector or pCMV-FLAG-LPA_2. The positive clones were pooled to establish RH7777-mock and RH7777-LPA_2 stable cell lines. The expression of the LPA_2 receptor mRNA was verified by RT-PCR analysis. The effect of LPA on the turnover rate of transfected HA-Siva-1 was determined as described above. To determine the effect of adriamycin on the turnover rate of Siva-1, these cells were treated with adriamycin for 20 h in serum-containing medium, and the turnover rate of Siva-1 was determined.

GST Pull-down Assay

To detect *in vitro* binding of Siva-1 and the carboxyl-terminal tail of the LPA_2 receptor, GST, GST-LPA_2-CT, or GST-Siva-1 was expressed in (BL21) (DE3) (LysS) *Escherichia coli* and purified by immobilizing the proteins on glutathione-Sepharose beads (Amersham Biosciences). GST-Siva-1 was further digested with PreScission Protease (Amersham Biosciences Bioscience) to cleave GST. 0.1 μg of Siva-1 was immediately incubated with 1 μg of GST or GST-LPA_2-CT for 2h at 4 °C. Siva-1 protein pulled down by GST-LPA_2-CT was detected with an anti-Siva-1 polyclonal antibody.

Semi-quantitative RT-PCR Analysis

NIH 3T3 cells transfected with different expression vectors were subjected to LPA or adriamycin treatment as indicated. Total RNA was extracted using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. Two to five μg of total RNA was subjected to RT-PCR analysis to amplify endogenous mouse Siva-1 (635 bp, primer sets: S1, 5′-CCATGCCCAAGCGGAGCTGCCC-3′ and S2, 5′-GCAAATATAAAGAGGTTTATTCA-3′), human Siva-1 (630 bp) or Siva-1-ΔC3 (440 bp) (primer sets: S1 and Siva-CT2: 5′-CCCGTCGACCCCACAGTTCAGTGTGAAACACCGA-3′), full-length LPA_2 receptor, and GAPDH (325 bp) (29), respectively. One-fourth to one-tenth of the RT mix was subjected to PCR amplification for 30 or 40 cycles. All of the conditions have been adjusted to ensure linear amplification of the mRNA.

Immunocytochemistry

To examine co-localization of Siva-1 with the LPA_2 receptor, GFP-Siva-1 was co-expressed with the FLAG-LPA_2 receptor or FLAG-LPA_2-ΔC in NIH 3T3 cells. The cells were starved overnight in 0.1% fatty acid-free BSA-containing medium. After fixation, permeabilization, and blocking, the cells were incubated with an anti-FLAG M2 monoclonal antibody (Sigma)

followed by the incubation with a Texas Red X-conjugated anti-mouse secondary antibody (Molecular Probes) to detect FLAG-LPA_2 receptor. The nuclei were stained with Hoechst 33258 (Sigma). The images of GFP and GFP-Siva-1 were directly captured by fluorescence microscopy (Axioplan 2, Zeiss). A similar procedure was performed to detect co-localization of FLAG-Siva-1 with HcRed1-LPA_2 or GFP-LPA_2 with HcRed1-Siva-1.

Adriamycin-induced Siva-1 Expression, Caspase-3 Cleavage, and Apoptosis

NIH 3T3 cells transiently expressing HA-Siva-1 or not were incubated in 0.1% fatty acid-free BSA-containing Dulbecco's modified Eagle's medium without or with 5 μM LPA for 6 h followed by the addition of 5 μM adriamycin for another 24 h. The cells were fixed in 70% ethanol and DNA was stained with propidium iodide (Roche Applied Science). The population of sub-G_1 cells was analyzed by flow cytometry (UAB Flow Cytometry Core Facility). Similar procedures were performed in NIH 3T3 cells overexpressing a scrambled siRNA, an LPA_2 siRNA, or a Siva-1 siRNA except that cells were pre-treated with 10 μM LPA for 6 h followed by the addition of 10 μM adriamycin for 20 h. Immunoblotting was performed to detect endogenous Siva-1, HA-Siva-1, procaspase-3, and β-actin using a Siva-1-specific polyclonal antibody, an anti-HA antibody (Santa Cruz Biotechnology), an anti-caspase-3 antibody (Santa Cruz Biotechnology), and an anti-β-actin antibody (Sigma), respectively. Apoptosis was also determined by annexin V-fluorescein isothiocyanate staining (BD Biosciences) followed by fluorescence-activated cell sorter analysis.

RESULTS

The Carboxyl-terminal Tail of the LPA_2 Receptor Interacts with the Carboxyl Cysteine-rich Domain of Siva-1 Protein

In an attempt to identify the molecules specifically involved in the regulation of the LPA_2 receptor, a fusion protein containing the carboxyl-terminal tail (aa 296–351) of the LPA_2 receptor (designated LPA_2-CT) and the Gal4 DNA-binding domain was used as bait to screen a HeLa cell cDNA library (14). Among the four million clones screened, one positive clone containing six base pairs 5′ to the translation start sites, the entire Siva-1 coding sequences, and the 3′-untranslated region was identified. The interaction has been verified by selective growth of yeast cells co-expressing full-length Siva-1 with LPA_2-CT, but not LPA_1-CT or LPA_3-CT, on plates lacking tryptophan, leucine, and histidine (Fig. 1*A*). To further examine a direct interaction between these two molecules, purified full-length Siva-1 was incubated with GST protein or GST fusion protein of LPA_2-CT *in vitro*. As shown in Fig. 1*B*, Siva-1 was pulled down by GST-LPA_2-CT but not GST, indicating a direct interaction between Siva-1 and the carboxyl-terminal tail of the LPA_2 receptor.

Previously we have shown that the carboxyl-terminal tail of the LPA_2 receptor directly interacts with the LIM domains of TRIP6 (14). In comparison with the two cysteine-rich zinc finger-like motifs (aa 114–170) of Siva-1, the second zinc finger motif of the TRIP6-LIM1 domain (aa 307–331) shares 32% identity with amino acids 114–138 of Siva-1, and the second zinc finger motif of the TRIP6-LIM3 domain (aa 432–460) shares 21% identity with amino acids 142–170 of Siva-1 (Fig. 1*C*). Given the structural similarity between the carboxyl cysteine-rich domains of Siva-1 and TRIP6, we speculated that the zinc finger-like motifs of Siva-1 might be responsible for LPA_2 receptor binding. To test this hypothesis, a number of cDNA constructs encoding GFP fusion proteins of Siva-1, Siva-2, and several Siva-1 deletion mutants (Fig. 1*D*) were generated and transfected without or with a Myc-LPA_2 receptor expression vector into HEK 293T cells. Because the expression of some Siva-1 mutants was very low, here the GFP fusion proteins of Siva-1 and Siva-1 deletion mutants were employed to ensure comparable expression levels of each protein. Fusion to GFP was able to increase the expression of Siva mutants, but GFP itself did not

nonspecifically associate with the Myc-LPA_2 receptor (Fig. 1*E*). Our result showed that the LPA_2 receptor co-immunoprecipitated with Siva-1, Siva-2, Siva-1-ΔN, Siva-1-CT, and Siva-1-ΔC1. In contrast, the receptor binding with either Siva-1-ΔC2 or Siva-1-ΔC3 was greatly diminished, indicating that the carboxyl cysteine-rich domain (aa 139–175) of Siva-1 is responsible for this interaction.

Next, we investigated whether Siva-1 interacts with the LPA_2 receptor at physiological levels. We reconstituted a FLAG-tagged human LPA_2 receptor in immortalized MEFs lacking both LPA_1 and LPA_2 receptors (designated DKO-LPA_2) and ensured that the reconstituted LPA_2 receptor was comparably expressed as the endogenous LPA_2 receptor in wild-type MEFs by semi-quantitative RT-PCR analysis (Fig. 2*A*, *left panel*). We found that endogenous Siva-1 specifically associated with the LPA_2 receptor in DKO-LPA_2 MEFs (Fig. 2*A*, *middle panel*); however, we could barely detect endogenous Siva-1 expression in the total lysates of DKO-LPA_2 cells (Fig. 2*A*, *middle panel*). Because Siva-1 mRNA was comparably expressed in DKO-mock and DKO-LPA_2 MEFs (Fig. 2*A*, *left panel*), we speculated that the stability of Siva-1 protein might be different in these two cell lines. Indeed, when both cell lines were incubated with MG-132 for 2 h to inhibit proteasomal degradation, they expressed similar levels of Siva-1 protein (Fig. 2*A*, *right panel*), and under this condition, endogenous Siva-1 was found to co-immunoprecipitate with the LPA_2 receptor in DKO-LPA_2 MEFs (Fig. 2*A*, *right panel*).

Previously we have shown that LPA stimulation rapidly recruits TRIP6 to the activated LPA_2 receptor but not other LPA receptors (14). To investigate whether Siva-1 also binds to the LPA_1 and LPA_3 receptors, co-immunoprecipitation of Siva-1 with different LPA receptors was performed in HEK 293T cells without or with LPA stimulation for 5 min. As shown in Fig. 2*B*, Siva-1 preferentially bound to the LPA_2 receptor. In contrast to TRIP6, Siva-1 associated with the LPA_2 receptor in the absence of serum, and this interaction was not affected by LPA stimulation for 5 min. Siva-1 also bound to the LPA_1 receptor with a much lower affinity but barely interacted with the LPA_3 receptor. Because the carboxyl-terminal tail of the LPA_1 receptor does not interact with Siva-1 (Fig. 1*A*), this result suggests that the LPA_1 receptor may bind to Siva-1 indirectly or through another weak binding site.

Although a brief stimulation with LPA has no effect on the association of Siva-1 with the LPA_2 receptor, prolonged LPA stimulation affects the expression and interaction of these two proteins. We found that in NIH 3T3 cells co-expressing GFP-Siva-1 and the FLAG-LPA_2 receptor, both Siva-1 and the LPA_2 receptor were expressed at much higher levels by serum starvation overnight (Fig. 2*C*, *lane 3* compared with *lane 1* and *lane 4* compared with *lane 2*). However, the expression of both proteins was reduced by further treatment with LPA for 2 h. Under this condition, similar amounts of Siva-1 were co-immunoprecipitated with the LPA_2 receptor compared with that in the absence of serum (Fig. 2*C*, *lane 5* compared with *lane 4*). This result may suggest that the association of Siva-1 and LPA_2 is increased by prolonged LPA treatment. Our result also showed that in serum-containing medium, Siva-1 was expressed at lower levels by co-expressed with the LPA_2 receptor (Fig. 2*C*, *lane 2* compared with *lane 1*). Because serum contains micro-molar concentrations of LPA (30), it is likely that the transfected LPA_2 receptor promotes LPA-induced reduction of Siva-1 protein in the presence of serum. Although TRIP6 also interacts with the LPA_2 receptor, the levels of TRIP6 were not affected by overexpression of the LPA_2 receptor.

We also found that LPA stimulation for 1 h attenuated the expression of LPA_1, LPA_2, and LPA_3 in HEK 293T cells (Fig. 2*D*). Although the LPA_1 receptor was able to bind to Siva-1 weakly (Fig. 2*B*), LPA reduced the expression of Siva-1 only when Siva-1 was co-expressed

with the LPA_2 receptor. Thus, these results suggest that LPA_2, but not LPA_1 or LPA_3, specifically regulates the expression of Siva-1 protein in an LPA-dependent manner.

The association of Siva-1 with the LPA_2 receptor was further confirmed by co-localization of GFP-Siva-1 with FLAG-LPA_2 (Fig. 3*A*) or HcRed1-Siva-1 with GFP-LPA_2 (Fig. 3*B*) in serum-free conditions and by co-localization of FLAG-Siva-1 with HcRed1-LPA_2 after LPA stimulation for 2 h (supplemental Fig. S1*C*). The result showed that Siva-1 was predominantly expressed in the nucleus, although it could also be found in the cytosol and plasma membrane (Fig. 3 and supplemental Fig. S1*A*). In contrast to Siva-1, the LPA_2 receptor was only present in the cytosol or on the plasma membrane (Fig. 3 and supplemental Fig. S1*B*). Strikingly, the cytosolic and membrane localization of Siva-1 was greatly increased in cells overexpressing the LPA_2 receptor, where Siva-1 appeared in clusters that coincided precisely with the distribution of the LPA_2 receptor (Fig. 3 and supplemental Fig. S1*C*). In contrast to the LPA_2 receptor, the LPA_2-ΔC mutant, which lacks the carboxyl-terminal tail to bind to Siva-1, failed to capture Siva-1 in the cytosol (Fig. 3*A*). This result suggests that the LPA_2 receptor binds to Siva-1 and prevents cytosolic Siva-1 from translocation into the nucleus.

To verify whether Siva-1 is indeed present in the nucleus, NIH 3T3 cells expressing Siva-1 without or with the LPA_2 receptor were treated with LPA for 1 h, and differential centrifugation was performed to separate nuclei and the supernatant, which contains subcellular organelles, cytosol and the plasma membrane. Consistently, the result showed that Siva-1 was predominantly present in the nuclear extract (supplemental Fig. S1*D*). When co-expressed with the LPA_2 receptor, the levels of Siva-1 were significantly lower and were further reduced by LPA.

The LPA_2 Receptor Promotes LPA-dependent Degradation of Siva-1 Protein

Many G protein-coupled receptors are endocytosed after ligand-induced activation (31). The internalized receptors are either recycled back to the plasma membrane in a process known as resensitization or undergo proteolytic degradation following prolonged ligand stimulation. Because the expression of LPA_2 receptor and Siva-1 was both reduced by prolonged LPA stimulation (Fig. 2, *C* and *D*), we speculated that both proteins might undergo LPA-dependent degradation concomitantly. Therefore, we set out to examine LPA-induced turnover of the LPA_2 receptor and Siva-1 in NIH 3T3 cells by pretreating cells with cycloheximide to inhibit protein synthesis. Our result showed that the turnover rate of the FLAG-LPA_2 receptor was enhanced by LPA stimulation in NIH 3T3 cells (Fig. 4*A*). Likewise, Siva-1 was more stable in serum-free conditions, whereas LPA induced a rapid turnover of endogenous Siva-1 (Fig. 4*B*) and transfected FLAG-Siva-1 (Fig. 4*C*). To investigate the effect of LPA_2 on Siva-1 turnover, next we examined the turnover of a transfected HA-Siva-1 in RH7777-mock rat hepatoma cells that do not express endogenous LPA_1, LPA_2, and LPA_3 (32) and in RH7777-LPA_2 cells stably expressing the FLAG-LPA_2 receptor. In general, the expression of Siva-1 was much lower in RH7777-LPA_2 cells than in RH7777-mock cells when equal amounts of Siva-1 cDNA were transfected (Fig. 4*D*). Our results showed that LPA treatment did not alter the expression of HA-Siva-1 in RH7777-mock cells but significantly attenuated its expression in RH7777-LPA_2 cells (Fig. 4*D*), suggesting that the LPA_2 receptor promotes LPA-induced Siva-1 degradation.

LPA Stimulation Increases Ubiquitination of Both Siva-1 Protein and the LPA_2 Receptor

Several G protein-coupled receptors have been shown to undergo ligand-dependent ubiquitination and degradation through the lysosomal or proteasomal pathways (33). We hypothesized that Siva-1 might undergo concomitant ubiquitination and degradation together with the LPA_2 receptor in response to LPA stimulation. To test this possibility,

HEK 293T cells expressing HA-ubiquitin with FLAG-LPA_2 or FLAG-Siva-1 were starved overnight and pretreated with MG-132 for 1 h before stimulation with LPA for another hour. Our result showed that ubiquitination of the LPA_2 receptor was found even in the absence of LPA (Fig. 5*A*). However, the levels of ubiquitinylated LPA_2 were increased by MG-132 pretreatment, which was further enhanced when followed by LPA stimulation (Fig. 5*A*). Similarly, LPA stimulation promoted Siva-1 ubiquitination, and the levels of ubiquitinylated Siva-1 were further increased by MG-132 (Fig. 5*B*). Using MG-132 to prevent protein degradation, we found that the ubiquitinylated Siva-1 and LPA_2 receptor were present in the same complex, and this complex formation was increased by LPA treatment for 60 min (Fig. 5*C*). Together, these results suggest that prolonged LPA stimulation promotes the association of Siva-1 with the LPA_2 receptor and targets both proteins for proteasomal ubiquitination and degradation.

DNA Damage-induced Siva-1 Stabilization Is Attenuated by LPA in an LPA_2 Receptor-dependent Manner

It has been shown that transcription of Siva-1 mRNA can be induced by treatment with the DNA damaging agents camptothecin and cisplatin (17, 18). Further evidence reveals that Siva-1 is a direct transcriptional target for p53 and E2F1 (17). Consistently, our result showed that adriamycin (Doxorubicin) transcriptionally activated Siva-1 mRNA in NIH 3T3 cells; however, this induction was not affected by LPA (Fig. 6*A*). Adriamycin not only induced endogenous Siva-1 protein expression but also stabilized the transfected Siva-1, particularly in serum-free conditions; however, this effect was attenuated by LPA pre-treatment (Fig. 6*B*).

The counteracting role of LPA in regulating adriamycin-mediated Siva-1 stability was further demonstrated by assessing the turnover rate of transfected FLAG-Siva-1 in NIH 3T3 cells treated with adriamycin and LPA (Fig. 6*C*). Because cycloheximide can facilitate apoptosis induction in some cases (34), here we also found that the addition of cycloheximide to inhibit protein synthesis further enhanced adriamycin-mediated stabilization of Siva-1 protein. Nonetheless, this effect was attenuated by LPA.

Next, we assessed the effect of LPA_2 receptor on the turnover of Siva-1 during DNA damage response. As shown in Fig. 6*D*, treatment with adriamycin and cycloheximide induced a robust activation and stabilization of Siva-1 in RH7777-mock cells but not RH7777-LPA_2 cells, suggesting a role for LPA_2 in mediating the down-regulation of Siva-1 during DNA damage response.

If the down-regulation of Siva-1 protein expression is mediated by LPA_2 binding, overexpression of the LPA_2 receptor should not alter the expression of Siva-1-ΔC3 mutant that lacks the LPA_2 receptor-interacting domain (Fig. 1*E*). To test this hypothesis, FLAG-Siva-1 or FLAG-Siva-1-ΔC3 was expressed without or with the Myc-LPA_2 receptor in NIH 3T3 cells, and then cells were treated with adriamycin for 20 h in serum-containing medium. Although the exogenous Siva-1 mRNA and Siva-1-ΔC3 mRNA were constitutively expressed, Siva-1 and Siva-1-ΔC3 proteins were significantly induced only when treated with adriamycin (Fig. 6*E*). This result suggests that adriamycin must have stabilized these proteins through the regulation of the amino-terminal region of Siva-1, which is independent on LPA_2 binding. However, only Siva-1, but not Siva-1-ΔC3, was attenuated by LPA_2 receptor overexpression, suggesting that the carboxyl cysteine-rich motif of Siva-1 mediates LPA_2 receptor-dependent Siva-1 degradation (Fig. 6*E*).

LPA Attenuates Siva-1 Function in Adriamycin-induced Apoptosis

It has been shown that overexpression of Siva-1 in itself is sufficient to activate caspase-3 activity and induce apoptosis in several cell lines (15, 21, 23–25). In contrast, LPA protects NIH 3T3 fibroblasts from serum starvation-induced apoptosis (7). Because LPA targets Siva-1 for degradation, it might block the proapoptotic function of Siva-1 during DNA damage response. Indeed, our results showed that inhibition of the LPA_2 receptor expression in NIH 3T3 cells increased Siva-1 levels and attenuated the protective effect of LPA against adriamycin-induced caspase-3 cleavage (Fig. 7*A*). Likewise, overexpression of Siva-1 significantly enhanced adriamycin-induced caspase-3 cleavage; however, this effect was reduced by LPA pre-treatment (Fig. 7*B*). Consistent with these observations, overexpression of an LPA_2 siRNA or Siva-1 protein promotes adriamycin-induced apoptosis (Fig. 7*C* and supplemental Fig. S2). In contrast, LPA protected cells from apoptosis in all of the transfected cells except for the LPA_2 siRNA-expressing cells, in which LPA showed a much less protective effect (Fig. 7*C*). Perhaps because adriamycin only induced a mild apoptosis in NIH 3T3 cells, knocking down the expression of Siva-1 either did not protect cells from adriamycin-induced caspase-3 cleavage (Fig. 7*B*) and apoptosis (annexin V staining; Fig. 7*C*) or only mildly reduced apoptosis (propidium iodide staining; supplemental Fig. S2*B*). Taken together, these results demonstrate that LPA not only serves as a survival factor but also plays an anti-apoptotic role by directly down-regulating the expression and pro-apoptotic activity of Siva-1 through a specific interaction of the LPA_2 receptor with Siva-1.

DISCUSSION

LPA is a growth factor-like phospholipid that has been shown to mediate cell survival through the activation of phosphatidylinositol 3-kinase-AKT and ERK signaling pathways and the induction of NF-κB gene expression (7–9, 12, 35). Our study further reveals a novel mechanism by which prolonged stimulation with LPA not only promotes ubiquitination and degradation of the LPA_2 receptor but also concomitantly down-regulates LPA_2-interacting Siva-1 protein. Consequently, the proapoptotic function of Siva-1 in DNA damage response is attenuated by LPA. Fig. 7*D* provides a model by which the interaction of Siva-1 with the LPA_2 receptor promotes LPA-dependent down-regulation of Siva-1 function during DNA damage response. Because the function of Siva-1 protein is important for inducing caspase-3 activity and apoptosis, an appropriate suppression of Siva-1 activity through LPA-induced Siva-1 protein degradation may serve as a mechanism for normal cell growth.

Previously it has been demonstrated that in primary chronic lymphocytic leukemia cells in which the LPA_1 receptor is up-regulated, LPA protects cells from spontaneous apoptosis through an LPA_1-dependent activation of AKT/PKB pathways (36). In contrast, in IEC-6 intestinal epithelial cells that express high levels of LPA_2 and lesser amounts of LPA_1, an LPA_2-selective agonist, FAP-12, protects cells from camptothecin-induced apoptosis (37). Moreover, another LPA_2 receptor-selective agonist, octadecenyl thiophosphate, specifically protects LPA_1-null mice, but not LPA_2-null mice, from radiation-induced apoptosis of intestinal epithelium (38). It remains to be tested whether octadecenyl thiophosphate elicits the anti-apoptotic effect through an LPA_2-dependent down-regulation of Siva-1 expression. Together, these findings suggest that LPA_1 and LPA_2 may utilize distinct anti-apoptotic signaling mechanisms to promote cell survival.

Thus far, a number of G protein-coupled receptors such as the β_2-adrenergic receptor, the CXCR4 chemokine receptor, the protease-activated receptor 2 and the V2 vasopressin receptor have been shown to undergo ubiquitination and degradation following prolonged agonist stimulation (39 – 42). In contrast, several other receptors such as the platelet-activating factor receptor, the opioid receptor, and the thyrotropin-releasing hormone receptor are ubiquitinated in an agonist-independent manner because of misfolding or

incomplete folding of the receptor during synthesis (42–44). Our results show that the LPA_2 receptor is ubiquitinated in the absence of ligand; however, in the presence of MG-132, LPA stimulation further promotes ubiquitination of the LPA_2 receptor. Thus far, only a very limited number of proteins that directly associate with the G protein-coupled receptors, such as β-Arrestin1, β-Arrestin2, and G protein-coupled receptor kinase 2, have been reported to undergo ligand-dependent ubiquitination and degradation (39, 45–47). Therefore, our results would provide another example of G protein-coupled receptor-mediated regulation of associated proteins through ligand-dependent ubiqutination and degradation. Whether LPA targets the LPA_2 receptor and Siva-1 to the same E3 ligase for ubiquitination remains to be determined.

Through the yeast two-hybrid screen, we have now identified Siva-1 and TRIP6 as the LPA_2 receptor-interacting proteins. The carboxyl-terminal cysteine-rich motif of Siva-1 and the second zinc finger motif of TRIP6-LIM3 domain share some structural similarity and are both important for the interaction with the LPA_2 receptor but not other LPA receptors. However, there are some functional discrepancies between these two molecules in LPA signaling. TRIP6 specifically interacts with the LPA_2 receptor upon LPA stimulation for 5–10 min. This association is probably transient because LPA treatment for 15–20 min induces the translocation of TRIP6 to focal adhesion plaques where the LPA_2 receptor is not present (14). In contrast, Siva-1 binds to the LPA_2 receptor in the absence of LPA (Fig. 2, *B* and *C*). However, using MG-132 to inhibit proteasomal degradation, we have demonstrated that LPA stimulation for 60 min promotes the complex formation of ubiquitinylated LPA_2 receptor and Siva-1 (Fig. 5*C*). This result suggests that Siva-1 may preferentially bind to the internalized LPA_2 receptor. The interaction of TRIP6 with the LPA_2 receptor promotes LPA-dependent association of TRIP6 with several focal adhesion molecules, thereby enhancing LPA-induced cell migration (14), whereas the interaction of Siva-1 with the LPA_2 receptor captures Siva-1 in the perinuclear region and promotes LPA-dependent degradation of Siva-1. In contrast to Siva-1, LPA stimulation for 60 min does not promote ubiquitination and degradation of TRIP6 (data not shown). This is possibly due to a transient interaction of TRIP6 with the LPA_2 receptor. It should be noted that our results do not exclude the possibility that LPA may mediate Siva-1 degradation through other transcriptional and post-translational mechanisms involved in cell survival and anti-apoptosis, and Siva-1 may be down-regulated by other serum factors in addition to LPA (Fig. 6*E*). Nonetheless, the interaction of Siva-1 with the LPA_2 receptor would provide a direct mechanism to promote LPA-induced Siva-1 degradation.

DNA damage response such as adriamycin treatment transcriptionally regulates Siva-1 gene expression through the activation of p53 and E2F1 (17) and also post-translationally stabilizes Siva-1 protein (Fig. 6). As a result, Siva-1 enhances DNA damage-induced apoptosis (Fig. 7, *B* and *C*). In addition to the LPA_2 receptor, Siva-1 has been shown to interact with two other cell surface receptors, including CD27 and GITR (the glucocorticoid-induced tumor necrosis factor receptor family-related gene) (15, 48). In contrast to the LPA_2 receptor that mediates LPA-induced cell survival and cell proliferation, CD27 and GITR are involved in T lymphocyte apoptosis (15, 49). Thus, these receptors may compete for Siva-1 binding in different cellular conditions. Intriguingly, Siva-1 shuttles between nucleus and cytosol and is present predominantly in the nucleus; however, all three Siva-1-interacting receptors are cell surface receptors. Moreover, Siva-1 induces apoptosis via a caspase-3-dependent mitochondrial pathway, although the detailed mechanism has yet to be elucidated. Whether nuclear Siva-1 plays an intrinsic role in apoptosis or has a completely different function remains to be explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Jun Xu for the technical assistance in yeast two-hybrid screening and Tracey McGuire at UAB Flow Cytometry Core Facility for the fluorescence-activated cell sorter analysis. We also thank Jason Paik for the critical reading of this manuscript. The anti-human LPA_2 antibody was a gift kindly provided by Dr. Anjaparavanda P. Naren. The immortalized double knock-out MEFs deficient in LPA_1 and LPA_2 were established by Drs. Natalia Makarova and Gabor Tigyi.

REFERENCES

1. Mills GB, Moolenaar WH. Nat. Rev. Cancer. 2003; 3:582–591. [PubMed: 12894246]
2. Noguchi K, Ishii S, Shimizu T. J. Biol. Chem. 2003; 278:25600–25606. [PubMed: 12724320]
3. An S, Bleu T, Huang W, Hallmark OG, Coughlin SR, Goetzl EJ. FEBS Lett. 1997; 417:279–282. [PubMed: 9409733]
4. Bandoh K, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H, Inoue K. J. Biol. Chem. 1999; 274:27776–27785. [PubMed: 10488122]
5. Hecht JH, Weiner JA, Post SR, Chun J. J. Cell Biol. 1996; 135:1071–1083. [PubMed: 8922387]
6. Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. J. Biol. Chem. 2006; 281:23589–23597. [PubMed: 16774927]
7. Fang X, Yu S, LaPushin R, Lu Y, Furui T, Penn LZ, Stokoe D, Erickson JR, Bast RC Jr. Mills GB. Biochem. J. 2000; 352:135–143. [PubMed: 11062066]
8. Radeff-Huang J, Seasholtz TM, Matteo RG, Brown JH. J. Cell Biochem. 2004; 92:949–966. [PubMed: 15258918]
9. Kang YC, Kim KM, Lee KS, Namkoong S, Lee SJ, Han JA, Jeoung D, Ha KS, Kwon YG, Kim YM. Cell Death Differ. 2004; 11:1287–1298. [PubMed: 15297884]
10. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Cell. 1997; 91:231–241. [PubMed: 9346240]
11. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Science. 1998; 282:1318–1321. [PubMed: 9812896]
12. Raj GV, Sekula JA, Guo R, Madden JF, Daaka Y. Prostate. 2004; 61:105–113. [PubMed: 15305333]
13. Xu Y, Shen Z, Wiper DW, Wu M, Morton RE, Elson P, Kennedy AW, Belinson J, Markman M, Casey G. J. Am. Med. Assoc. 1998; 280:719–723.
14. Xu J, Lai YJ, Lin WC, Lin FT. J. Biol. Chem. 2004; 279:10459–10468. [PubMed: 14688263]
15. Prasad KV, Ao Z, Yoon Y, Wu MX, Rizk M, Jacquot S, Schlossman SF. Proc. Natl. Acad. Sci. U. S. A. 1997; 94:6346–6351. [PubMed: 9177220]
16. Daoud SS, Munson PJ, Reinhold W, Young L, Prabhu VV, Yu Q, LaRose J, Kohn KW, Weinstein JN, Pommier Y. Cancer Res. 2003; 63:2782–2793. [PubMed: 12782583]
17. Fortin A, MacLaurin JG, Arbour N, Cregan SP, Kushwaha N, Callaghan SM, Park DS, Albert PR, Slack RS. J. Biol. Chem. 2004; 279:28706–28714. [PubMed: 15105421]
18. Qin LF, Lee TK, Ng IO. Life Sci. 2002; 70:1677–1690. [PubMed: 11991255]
19. Xiao H, Palhan V, Yang Y, Roeder RG. EMBO J. 2000; 19:956–963. [PubMed: 10698937]
20. Jacobs SB, Basak S, Murray JI, Pathak N, Attardi LD. Cell Death Differ. 2007; 14:1374–1385. [PubMed: 17464332]
21. Py B, Slomianny C, Auberger P, Petit PX, Benichou S. J. Immunol. 2004; 172:4008–4017. [PubMed: 15034012]
22. Gudi R, Barkinge J, Hawkins S, Chu F, Manicassamy S, Sun Z, Duke-Cohan JS, Prasad KV. Oncogene. 2006; 25:3458–3462. [PubMed: 16491128]
23. Xue L, Chu F, Cheng Y, Sun X, Borthakur A, Ramarao M, Pandey P, Wu M, Schlossman SF, Prasad KV. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:6925–6930. [PubMed: 12011449]

24. Yoon Y, Ao Z, Cheng Y, Schlossman SF, Prasad KV. Oncogene. 1999; 18:7174–7179. [PubMed: 10597319]
25. Cao C, Ren X, Kharbanda S, Koleske A, Prasad KV, Kufe D. J. Biol. Chem. 2001; 276:11465–11468. [PubMed: 11278261]
26. Liu K, Paik JC, Wang B, Lin FT, Lin WC. EMBO J. 2006; 25:4795–4807. [PubMed: 17006541]
27. Contos JJ, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J. Mol. Cell Biol. 2002; 22:6921–6929. [PubMed: 12215548]
28. Li C, Dandridge KS, Di A, Marrs KL, Harris EL, Roy K, Jackson JS, Makarova NV, Fujiwara Y, Farrar PL, Nelson DJ, Tigyi GJ, Naren AP. J. Exp. Med. 2005; 202:975–986. [PubMed: 16203867]
29. Wang B, Liu K, Lin FT, Lin WC. J. Biol. Chem. 2004; 279:54140–54152. [PubMed: 15494392]
30. Baker DL, Desiderio DM, Miller DD, Tolley B, Tigyi GJ. Anal. Biochem. 2001; 292:287–295. [PubMed: 11355863]
31. Drake MT, Shenoy SK, Lefkowitz RJ. Circ. Res. 2006; 99:570–582. [PubMed: 16973913]
32. Fukushima N, Kimura Y, Chun J. Proc. Natl. Acad. Sci. U. S. A. 1998; 95:6151–6156. [PubMed: 9600933]
33. Wojcikiewicz RJ. Trends Pharmacol. Sci. 2004; 25:35–41. [PubMed: 14723977]
34. Martin SJ, Lennon SV, Bonham AM, Cotter TG. J. Immunol. 1990; 145:1859–1867. [PubMed: 2167911]
35. Ye X, Ishii I, Kingsbury MA, Chun J. Biochim. Biophys. Acta. 2002; 1585:108–113. [PubMed: 12531543]
36. Hu X, Haney N, Kropp D, Kabore AF, Johnston JB, Gibson SB. J. Biol. Chem. 2005; 280:9498–9508. [PubMed: 15618220]
37. Virag T, Elrod DB, Liliom K, Sardar VM, Parrill AL, Yokoyama K, Durgam G, Deng W, Miller DD, Tigyi G. Mol. Pharmacol. 2003; 63:1032–1042. [PubMed: 12695531]
38. Deng W, Shuyu E, Tsukahara R, Valentine WJ, Durgam G, Gududuru V, Balazs L, Manickam V, Arsura M, VanMiddlesworth L, Johnson LR, Parrill AL, Miller DD, Tigyi G. Gastroenterology. 2007; 132:1834–1851. [PubMed: 17484878]
39. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Science. 2001; 294:1307–1313. [PubMed: 11588219]
40. Marchese A, Benovic JL. J. Biol. Chem. 2001; 276:45509–45512. [PubMed: 11641392]
41. Martin NP, Lefkowitz RJ, Shenoy SK. J. Biol. Chem. 2003; 278:45954–45959. [PubMed: 12960162]
42. Dupre DJ, Chen Z, Le Gouill C, Theriault C, Parent JL, Rola-Pleszczynski M, Stankova J. J. Biol. Chem. 2003; 278:48228–48235. [PubMed: 14500726]
43. Petaja-Repo UE, Hogue M, Laperriere A, Bhalla S, Walker P, Bouvier M. J. Biol. Chem. 2001; 276:4416–4423. [PubMed: 11054417]
44. Cook LB, Zhu CC, Hinkle PM. Mol. Endocrinol. 2003; 17:1777–1791. [PubMed: 12805411]
45. Shenoy SK, Lefkowitz RJ. J. Biol. Chem. 2003; 278:14498–14506. [PubMed: 12574160]
46. Penela P, Ruiz-Gomez A, Castano JG, Mayor F Jr. J. Biol. Chem. 1998; 273:35238–35244. [PubMed: 9857063]
47. Salcedo A, Mayor F Jr. Penela P. EMBO J. 2006; 25:4752–4762. [PubMed: 17006543]
48. Spinicelli S, Nocentini G, Ronchetti S, Krausz LT, Bianchini R, Riccardi C. Cell Death Differ. 2002; 9:1382–1384. [PubMed: 12478477]
49. Nocentini G, Giunchi L, Ronchetti S, Krausz LT, Bartoli A, Moraca R, Migliorati G, Riccardi C. Proc. Natl. Acad. Sci. U. S. A. 1997; 94:6216–6221. [PubMed: 9177197]

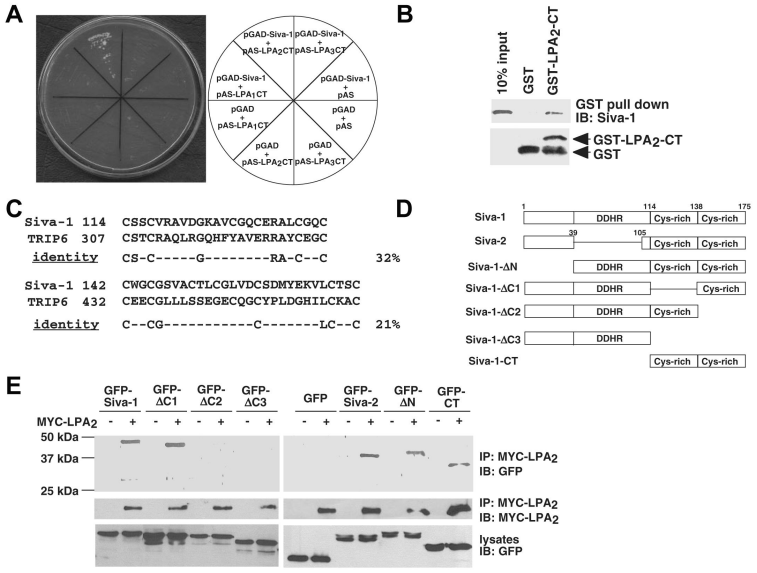

FIGURE 1. The carboxyl cysteine-rich domain of Siva-1 interacts with the carboxyl-terminal tail of the LPA_2 receptor *in vitro* and in cells

A, Siva-1 interacts with LPA_2-CT, but not LPA_1-CT or LPA_3-CT in yeast. The pAS2–1 expression vector of LPA_1-CT, LPA_2-CT or LPA_3-CT (14) was transformed into yeast Y190 cells with either pGAD or pGAD-Siva-1. The interaction of Siva-1 with LPA_2-CT was verified by selective growth of transformants on a plate lacking tryptophan, leucine, and histidine supplemented with 3-amino-1,2,4-triazole. *B*, Siva-1 interacts with the carboxyl-terminal tail of the LPA_2 receptor *in vitro*. Purified recombinant Siva-1 was incubated with GST or GST-LPA_2-CT at 4 °C for 2 h. Siva-1 pulled down by GST-LPA_2-CT was detected by immunoblotting (*IB*) with an anti-Siva-1 antibody. GST-LPA_2 and GST were detected with a GST-specific antibody. The *first lane* shows 10% input of Siva-1 used in this experiment. *C*, sequence comparison between the cysteine-rich region (aa 114–138 and 142–170) of Siva-1 and the second zinc finger motifs of LIM1 and LIM3 domains (aa 307–331 and 432–460) of TRIP6. *D*, the schematic structures of Siva-1, Siva-2, and different deletion mutants of Siva-1. Siva-1 contains a death domain homology region (*DDHR*, aa 48–114) and two cysteine-rich domains (aa 114–138 and 142–175). The amino acids 40–104 are absent in Siva-2. *E*, the carboxyl cysteine-rich domain of Siva-1 interacts with the LPA_2 receptor in HEK 293T cells. GFP fusion proteins of Siva-1, Siva-2, and different deletion mutants of Siva-1 were expressed without or with the Myc-LPA_2 receptor in HEK 293T cells. The Myc-LPA_2 receptor was immunoprecipitated (*IP*) with anti-Myc (9E10) antibody-conjugated agarose beads, and the immunoblot (*IB*) was probed with an anti-GFP polyclonal antibody to detect co-immunoprecipitated GFP fusion proteins of Siva-1 or Siva-1 mutants. The same blot was stripped and reprobed with an anti-Myc polyclonal antibody to detect immunoprecipitated receptors. The result shown is the LPA_2 receptor monomer (~37 kDa). The *bottom panel* is the expression of GFP, GFP-Siva-1, and GFP-Siva-1 mutants in the whole cell lysates. The result shown in *A*, *B*, or *E* is a representative from three independent experiments.

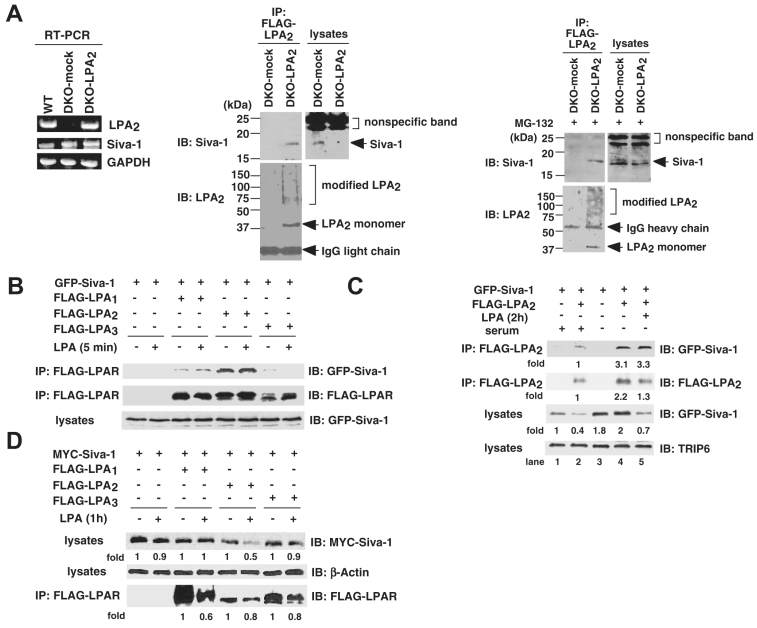

FIGURE 2. Siva-1 interacts with the LPA_2 receptor in cells

A, the LPA_2 receptor interacts with endogenous Siva-1 in $lpa_1^{-/-}$, $lpa_2^{-/-}$ double knock-out fibroblasts stably expressing a human FLAG-LPA_2. pCMV-FLAG-LPA_2 or pCMV-FLAG was stably transfected into immortalized MEFs deficient in LPA_1 and LPA_2 (designated DKO-LPA_2 and DKO-mock, respectively). The *left panel* is a semi-quantitative RT-PCR analysis showing the mRNA expression of LPA_2, Siva-1 and GAPDH. In the *middle panel*, the LPA_2 receptor was immunoprecipitated (*IP*) with anti-FLAG M2 monoclonal antibody-conjugated agarose beads from 5-mg lysates of DKO-LPA_2 or DKO-mock cells. The immunoblot (*IB*) was probed with an anti-Siva-1 polyclonal antibody to detect co-immunoprecipitated endogenous Siva-1. The blot was then reprobed with an anti-LPA_2 polyclonal antibody. The result shown is the ~37-kDa LPA_2 monomer and the modified forms of LPA_2 (~75–200 kDa). In the *right panel*, DKO-mock and DKO-LPA_2 cells were treated with MG-132 for 2 h. Co-immunoprecipitation of endogenous Siva-1 and the reconstituted FLAG-LPA_2 receptor was performed as described above. *B*, Siva-1 predominantly associated with the LPA_2 receptor in HEK 293T cells. GFP-Siva-1 was co-expressed with one of the FLAG-LPA receptors in HEK 293T cells as indicated. The cells were starved overnight followed by the incubation with LPA for 5 min. The LPA receptors were immunoprecipitated with anti-FLAG M2 monoclonal antibody-conjugated agarose beads, and the immunoblot was probed with an anti-GFP polyclonal antibody to detect co-immunoprecipitated GFP-Siva-1. The blot was stripped and reprobed with an anti-FLAG polyclonal antibody to detect the LPA receptors. The result shown is the LPA receptor monomer (~37–40 kDa). The *bottom panel* shows the expression of GFP-Siva-1 in the whole cell lysates. *C*, prolonged LPA stimulation regulates the expression and association of the LPA_2 receptor and Siva-1. NIH 3T3 cells expressing GFP-Siva-1 alone or GFP-Siva-1 with the FLAG-LPA_2 receptor were split evenly into different plates. The cells were either cultured in serum-containing medium or starved overnight followed by the incubation with LPA for 2 h. Co-immunoprecipitation of the FLAG-LPA_2 receptor with GFP-Siva-1 was performed as described above. The expression of GFP-Siva-1 and TRIP6 in the total lysates was detected with an anti-GFP polyclonal antibody and an anti-TRIP6 monoclonal antibody, respectively. The levels of GFP-Siva-1 and FLAG-LPA_2 were quantified by NIH IMAGE J software and normalized by the intensity of TRIP6. *D*, the levels of Siva-1 were reduced by prolonged LPA stimulation only when it was co-expressed with the LPA_2 receptor, but not

LPA_1 or LPA_3 receptor. HEK 293T cells transiently expressing Myc-Siva-1 with either FLAG-tagged LPA_1, LPA_2, or LPA_3 were starved overnight followed by the incubation with LPA for 1 h. The results shown are the Myc-Siva-1 and β-actin in the total lysates and the FLAG-LPA receptor monomer immunoprecipitated from the same amounts of total lysates. The intensity of Myc-Siva-1 and FLAG-LPA receptors was quantified and normalized by the intensity of β-actin and was compared without or with LPA treatment in each set. The result shown in each figure is a representative from three independent experiments. *WT*, wild type.

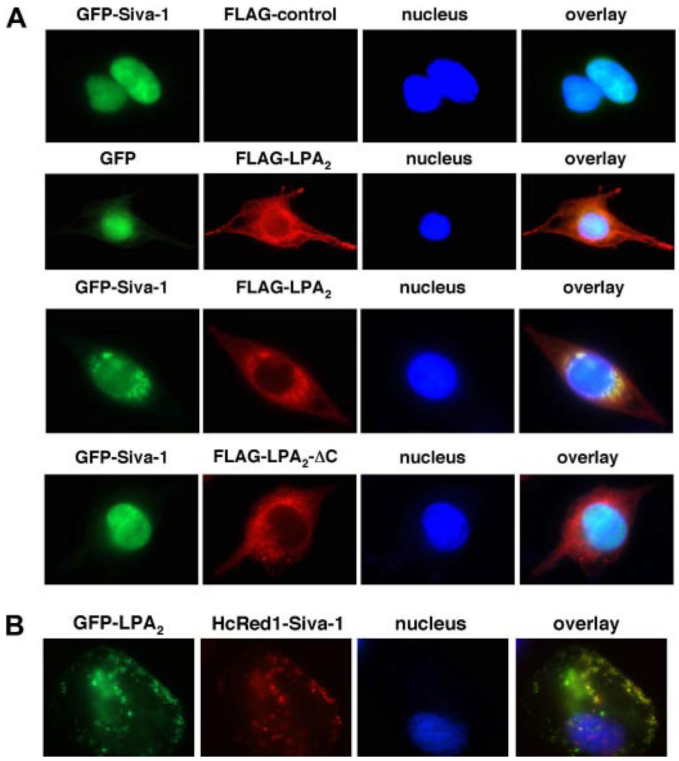

FIGURE 3. Siva-1 is predominantly present in the nucleus but co-localizes with the LPA_2 receptor in the cytosol or on the plasma membrane

A, GFP-Siva-1 or GFP was transiently expressed with the FLAG-LPA_2 receptor or FLAG-LPA_2-ΔC mutant in NIH 3T3 cells as indicated. The cells were starved overnight and then fixed. The images of GFP and GFP-Siva-1 were captured directly by fluorescence microscopy. FLAG-LPA_2 or FLAG-LPA_2-ΔC was detected by immunostaining with an anti-FLAG M2 monoclonal antibody followed by a Texas Red X-conjugated anti-mouse secondary antibody. *B*, GFP-LPA_2 and HcRed1-Siva-1 were co-expressed in NIH 3T3 cells. The images were directly visualized by fluorescence microscopy.

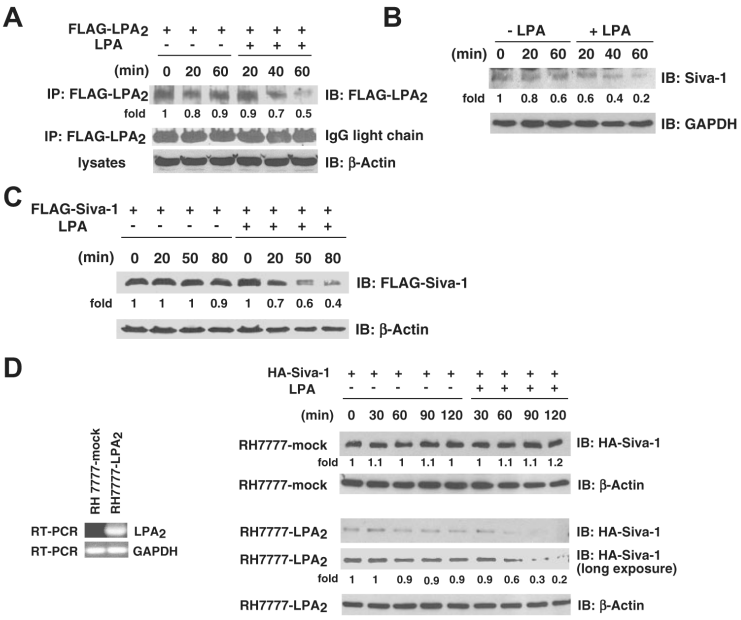

FIGURE 4. LPA promotes the turnover of Siva-1 and the LPA_2 receptor

A, LPA promotes the turnover of the LPA_2 receptor. NIH 3T3 cells expressing FLAG-LPA_2 were starved for 2 h and pretreated with cycloheximide for 5 min before the incubation with LPA as indicated. The result shown is the immunoprecipitated (*IP*) LPA_2 monomer, mouse IgG light chain control, and the expression of β-actin in the total lysates as a loading control. *B*, LPA promotes the turnover of Siva-1 protein. NIH 3T3 cells were starved for 2 h and pretreated with cycloheximide for 5 min before the incubation with LPA as indicated. The expression of endogenous Siva-1 in the whole cell lysates was detected with an anti-Siva-1 polyclonal antibody. The same blot was reprobed with an anti-GAPDH antibody. *C*, the transfected Siva-1 protein was stable in serum-free conditions but was rapidly degraded by LPA stimulation. The turnover rate of FLAG-Siva-1 was determined in NIH 3T3 cells transiently expressing FLAG-Siva-1 as described above. The blot was probed with an anti-FLAG antibody followed by an anti-β-actin antibody. *D*, LPA induces a rapid turnover of Siva-1 protein in RH7777-LPA_2 cells but not in RH7777-mock cells. HA-Siva-1 was expressed in RH7777-mock or RH7777-LPA_2 cells. The turnover rate of HA-Siva-1 was determined as described above. The expression HA-Siva-1 in the total lysates was detected with an anti-HA polyclonal antibody. The *left panel* shows the mRNA expression of human FLAG-LPA_2 and GAPDH in RH7777-mock and RH7777-LPA_2 cells by RT-PCR analysis. The intensity of proteins shown in each figure was quantified, compared with that shown in time zero, and normalized by the intensity of GAPDH or β-actin in each sample. The data shown in each figure is a representative result from three independent experiments. *IB*, immunoblot.

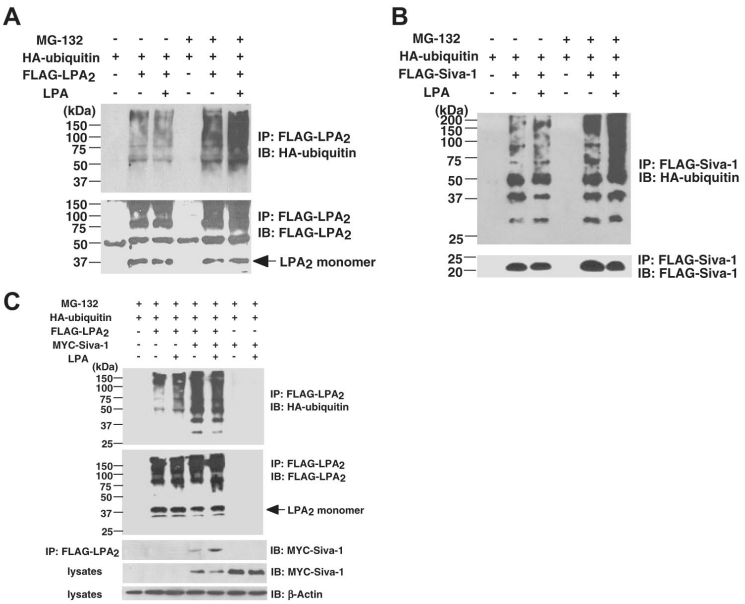

FIGURE 5. LPA induces ubiquitination of the LPA_2 receptor and Siva-1 and promotes the complex formation of ubiquitinylated LPA_2 receptor and Siva-1

The HA-ubiquitin expression vector was co-transfected with the vector(s) expressing FLAG-LPA_2 receptor (*A*), FLAG-Siva-1 (*B*), Myc-Siva-1, and the FLAG-LPA_2 receptor (*C*) into HEK 293T cells as indicated. The cells were starved overnight, pretreated with MG-132 for 1 h, and then incubated with LPA for another hour. FLAG-tagged Siva-1 or LPA_2 receptor was immunoprecipitated (*IP*) with anti-FLAG M2 antibody-conjugated agarose beads, and the immunoblot (*IB*) was probed with an anti-HA polyclonal antibody to detect ubiquitinylated LPA_2 receptor or Siva-1. The same blot was stripped and reprobed with an anti-FLAG polyclonal antibody to detect the FLAG-LPA_2 receptor (*A*) or FLAG-Siva-1 (*B*). Myc-Siva-1 co-immunoprecipitated with the FLAG-LPA_2 receptor was detected with an anti-Myc antibody (*C*).

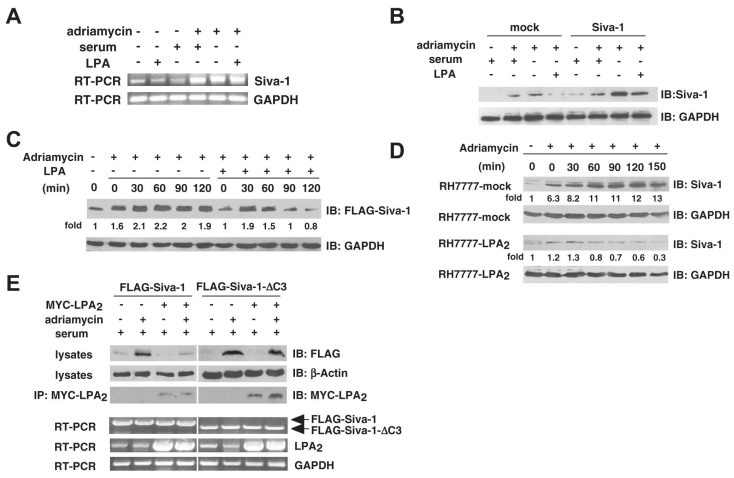

FIGURE 6. Adriamycin-induced Siva-1 protein stabilization is attenuated by LPA in an LPA_2 receptor-dependent manner

A, adriamycin-induced Siva-1 mRNA expression is not affected by LPA. NIH 3T3 cells were incubated with 5 μM LPA and/or 5 μM adriamycin in 0.1% fatty acid-free BSA-containing medium or serum-containing medium for 16h as indicated. Semi-quantitative RT-PCR analysis was performed to determine the mRNA levels of Siva-1 and GAPDH. *B*, adriamycin-induced Siva-1 protein stabilization is attenuated by LPA pretreatment. NIH 3T3 cells transfected with Siva-1 or not were pretreated with 10 μM LPA in 0.1% fatty acid-free BSA-containing medium for 6 h followed by the addition of 10 μM adriamycin for another 20 h. Immunoblotting (*IB*) was performed to determine the protein levels of Siva-1 and GAPDH in the whole cell lysates. The relative fold difference of Siva-1 expression was quantified as described above. *C*, LPA plays a counteracting role in regulating the turnover rate of Siva-1 during DNA damage response. NIH 3T3 cells transiently expressing FLAG-Siva-1 were pretreated with LPA or not for 6 h followed by the addition of adriamycin for another 20 h. The turnover rate of FLAG-Siva-1 was determined as described above. *D*, adriamycin induces and stabilizes Siva-1 in RH7777 cells, whereas this effect is greatly reduced in RH7777 cells stably expressing the LPA_2 receptor. RH7777-mock and RH7777-LPA_2 cells were treated with 10 μM adriamycin for 20 h in serum-containing medium. After cycloheximide pretreatment for 5 min, the turnover rate of Siva-1 was determined as described above. *E*, adriamycin-mediated stabilization of Siva-1, but not Siva-1-ΔC3, is attenuated by overexpression of the LPA_2 receptor. FLAG-Siva-1 or FLAG-Siva-1-ΔC3 was expressed alone or co-expressed with the Myc-LPA_2 receptor in NIH 3T3 cells. The cells were treated with 5 μM adriamycin for 20 h in serum-containing medium. The expression of FLAG-Siva-1 in 30 μg of cell lysates or FLAG-Siva-1-ΔC3 in 150 μg of cell lysates was detected by immunoblotting with an anti-FLAG antibody, and the blot was reprobed with an anti-β-actin antibody. The Myc-LPA_2 receptor monomer was detected as described in Fig. 1*E*. The *bottom three panels* show the mRNA expression of FLAG-Siva-1, FLAG-Siva-1-ΔC3, total LPA_2 receptors, and GAPDH. The data shown in each figure are representative results from three independent experiments.

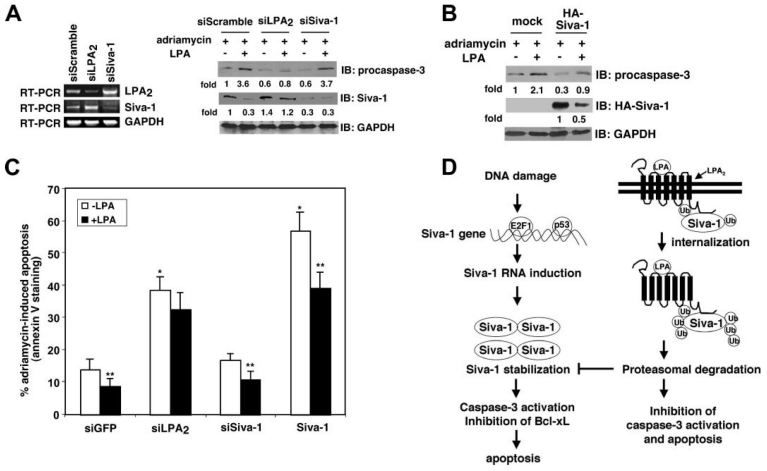

FIGURE 7. LPA attenuates Siva-1 function in adriamycin-induced caspase-3 cleavage and apoptosis

A, the effects of LPA2 siRNA and Siva-1 siRNA on the expression of Siva-1 and caspase-3 cleavage in cells treated with adriamycin and LPA. NIH 3T3 cells transiently expressing a scrambled siRNA (*siScramble*), LPA_2 siRNA (*siLPA*$_2$), or Siva-1 siRNA (*siSiva-1*) were pretreated with LPA for 6 h followed by the incubation with adriamycin for 20 h. The cell lysates were subjected to immunoblotting (*IB*) using an antibody specific to Siva-1, caspase-3, or GAPDH, respectively. *B*, Siva-1 augments adriamycin-induced caspase-3 cleavage, whereas this function is inhibited by LPA. NIH 3T3 cells expressing HA-Siva-1 or not were pretreated with LPA for 6 h followed by the incubation with adriamycin for another 20 h. Immunoblotting was performed to determine the levels of HA-Siva-1, procaspase-3, and GAPDH in the total lysates. *C*, inhibition of the LPA_2 receptor expression or overexpression of Siva-1 promotes adriamycin-induced apoptosis. NIH 3T3 cells expressing a control GFP siRNA (*siGFP*), LPA_2 siRNA, Siva-1 siRNA, or Siva-1 protein were pretreated with LPA for 6 h followed by the incubation with adriamycin for 20 h. Apoptosis was determined by annexin V-fluorescein isothiocyanate staining followed by fluorescence-activated cell sorter analysis. The data shown are the means ± S.E. of three to four independent experiments. *, $p < 0.05$ compared with the siGFP control with adriamycin treatment. **, $p < 0.05$ compared with that without LPA pretreatment in each set of samples, Student's *t* test. *D*, a model for the LPA_2 receptor-dependent down-regulation of Siva-1 to promote cell survival.