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

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Lyosphosphatidic acid (LPA) 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 LPA1, LPA2, and LPA3 receptors belong to the endothelial differentiation gene family, whereas the LPA4 and LPA5 receptors are structurally distinct from 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 LPA2 receptor as bait in a yeast two-hybrid screen to identify molecules specifically involved in the function of the LPA2 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 LPA2 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. 1D) (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 LPA2 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 down-regulates the proapoptotic activity of Siva-1 through specific binding of the LPA2 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′ untranslated region, was cloned into pscDNA3 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 pscDNA3-HA expression vector, respectively, such that these proteins were tagged in-frame with a FLAG epitope, a Myc epitope, or pcDNA3-HA expression vector, NIH 3T3 cells transiently expressing Siva-1 without or with LPA2 receptor were starved in 0.1% fatty acid BSA-containing medium overnight followed by the incubation with 2 μM LPA for 1 h and then harvested for subcellular fractionation in hypotonic solution (20 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 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-vinulcin 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 LPA2 receptor, NIH 3T3 cells were transfected with vectors expressing FLAG-Siva-1 or FLAG-LPA2 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 and the LPA2 receptor were detected by immunoprecipitation using an anti-FLAG polyclonal antibody (Santa Cruz Biotechnology) to detect ubiquitinylated proteins. To determine whether Siva-1 is expressed in the nucleus, NIH 3T3 cells transiently expressing Siva-1 without or with LPA2 receptor were starved in 0.1% fatty acid BSA-containing medium overnight followed by the incubation with 2 μM LPA for 1 h and then harvested for subcellular fractionation in hypotonic solution (20 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 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-vinulcin antibody (BD Biosciences) and an anti-histone antibody (Roche Applied Science) as a cytosolic and nuclear marker, respectively.

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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-LPA2. The positive clones were pooled to establish RH7777-mock and RH7777-LPA2 stable cell lines. The expression of the LPA2...
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 LPA₂ receptor, GST, GST-LPA₂-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 (Amer sham Biosciences Bioscience) to cleave GST. 0.1 µg of Siva-1 was immediately incubated with 1 µg of GST or GST-LPA₂-CT for 2 h at 4°C. Siva-1 protein pulled down by GST-LPA₂-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’-CCATGCCCAACGGAGCTGCCC-3’ and S2, 5’-GCAAATATAAAGAGGTTTATTCA-3’), human Siva-1 (630 bp) or Siva-1-ΔC (440 bp) (primer sets: S1 and Siva-CT2: 5’-CCCGTCGACCCCAAGCTTACTAACACACC-GA-3’), full-length LPA₂ 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 LPA₂ receptor, GFP-Siva-1 was co-expressed with the FLAG-LPA₂ receptor or FLAG-LPA₂-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-LPA₂ 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-LPA₂ or GFP-LPA₂ 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-G₁ cells was analyzed by flow cytometry (UAB Flow Cytometry Core Facility). Similar procedures were performed in NIH 3T3 cells overexpressing a scrambled siRNA, an LPA₂ siRNA, or a Siva-1 siRNA except that cells were pretreated 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 anticaspase-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 LPA₂ 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 LPA₂ receptor, a fusion protein containing the carboxyl-terminal tail (aa 296–351) of the LPA₂ receptor (designated LPA₂-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 site, the entire Siva-1 coding sequences, and the 3’ untranslated region was identified. The interaction was verified by selective growth of yeast cells co-expressing full-length Siva-1 with LPA₂-CT, but not LPA₂-CT or LPA₂-CT on plates lacking tryptophan, leucine, and histidine (Fig. 1A). 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 LPA₂-CT in vitro. As shown in Fig. 1B, Siva-1 was pulled down by GST-LPA₂-CT but not GST, indicating a direct interaction between Siva-1 and the carboxyl-terminal tail of the LPA₂ receptor.

Previously we have shown that the carboxyl-terminal tail of the LPA₂ 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 342–360) shares 21% identity with amino acids 142–170 of Siva-1 (Fig. 1C). 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 LPA₂ 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. 1D) were generated and transfected without or with a Myc-LPA₂ 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-LPA₂ receptor (Fig. 1E). Our result showed that the LPA₂ 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 LPA₂ receptor at physiological levels. We reconstituted a FLAG-
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**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 result shown in E is a representative from three independent experiments.

**TABLE 1.**

|     | GFP-Siva-1 | GFP-ΔC1 | GFP-ΔC2 | GFP-ΔC3 |
|-----|------------|---------|---------|---------|
| 50 kDa | - | - | - | + |
| 37 kDa | + | - | + | + |
| 25 kDa | - | + | + | + |

**TABLE 2.**

|     | IP: MYC-LPA$_2$ | IB: GFP |
|-----|----------------|---------|
| Siva-1 | - | - |
| Siva-2 | - | - |
| Siva-1-ΔN | - | - |
| Siva-1-ΔC1 | - | - |
| Siva-1-ΔC2 | - | - |
| Siva-1-ΔC3 | - | - |
| Siva-1-CT | - | - |

**Legend:**

- IP: Immunoprecipitation
- IB: Immunoblotting

- Cys-rich: Cysteine-rich domain
- DDHR: Death domain homology region

**Results:**

- Tagged human LPA$_2$ receptor in immortalized MEFs lacking both LPA$_1$ and LPA$_2$ receptors (designated DKO-LPA$_{2,1}$) 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. 2A, left panel). We found that endogenous Siva-1 specifically associated with the LPA$_2$ receptor in DKO-LPA$_{2,1}$ MEFs (Fig. 2A, middle panel); however, we could barely detect endogenous Siva-1 expression in the total lysates of DKO-LPA$_2$ cells (Fig. 2A, middle panel). Because Siva-1 mRNA was comparably expressed in DKO-mock and DKO-LPA$_2$ MEFs (Fig. 2A, 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. 2A, 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. 2A, right panel).

- Previously we have shown that LPA stimulation rapidly recruits TRIP$_6$ 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...
Siva-1 interacts with the LPA₂ receptor in cells. A, the LPA₂ receptor interacts with endogenous Siva-1 in \(lpa_{1/-}, lpa_{2/-}\) double knock-out fibroblasts stably expressing a human FLAG-LPA₂. pCMV-FLAG-LPA₂ or pCMV-FLAG was stably transfected into immortalized MEFs deficient in LPA₁ and LPA₂ (designated DKO-LPA₂ and DKO-mock, respectively). The left panel is a semi-quantitative RT-PCR analysis showing the mRNA expression of LPA₂, Siva-1 and GAPDH. In the middle panel, the LPA₂ receptor was immunoprecipitated (IP) with anti-FLAG M2 monoclonal antibody-conjugated agarose beads from 5-mg lysates of DKO-LPA₂ 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-LPA₂ polyclonal antibody. The result shown is the ~37-kDa LPA₂ monomer and the modified forms of LPA₂ (~75–200 kDa). In the right panel, DKO-mock and DKO-LPA₂ cells were treated with MG-132 for 2 h. Co-immunoprecipitation of endogenous Siva-1 and the reconstituted FLAG-LPA₂ receptor was performed as described above. B, Siva-1 predominantly associated with the LPA₂ 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 LPA₂ receptor and Siva-1. NIH 3T3 cells expressing GFP-Siva-1 alone or GFP-Siva-1 with the FLAG-LPA₂ 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-LPA₂ 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 LPA₂ 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 LPA₂ receptor, but not LPA₁ or LPA₃ receptor. HEK 293T cells transiently expressing Myc-Siva-1 with either FLAG-tagged LPA₁, LPA₂, or LPA₃ were starved overnight followed by the incubation with LPA for 1 h. The results shown are the Myc-Siva-1 and \(\beta\)-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 \(\beta\)-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.

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Fig. 2B, Siva-1 preferentially bound to the LPA₂ receptor. In contrast to TRIP6, Siva-1 associated with the LPA₂ receptor in the absence of serum, and this interaction was not affected by LPA stimulation for 5 min. Siva-1 also bound to the LPA₃ receptor with a much lower affinity but barely interacted with the LPA₁ receptor. Because the carboxyl-terminal tail of the LPA₂ receptor does not interact with Siva-1 (Fig. 1A), this result suggests that the LPA₂ 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 LPA₂ receptor, prolonged LPA stimulation affects the expression and interaction of these two
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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. 2C, 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. 2C, lane 5 compared with lane 4). This result may suggest that the association of Siva-1 and the 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. 2C, lane 2 compared with lane 1). Because serum contains micromolar 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. 2D). Although the LPA$_1$ receptor was able to bind to Siva-1 weakly (Fig. 2B), 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. 3A) or HcRed1-Siva-1 with GFP-LPA$_2$ (Fig. 3B) in serum-free conditions and by co-localization of FLAG-Siva-1 with HcRed1-LPA$_2$ after LPA stimulation for 2 h (supplemental Fig. S1C). 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. S1A). 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. S1B). 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. S1C). 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. 3A). 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. S1D). 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. 4A). Likewise, Siva-1 was more stable in serum-free conditions, whereas LPA induced a rapid turnover of endogenous Siva-1 (Fig. 4B) and transfected FLAG-Siva-1 (Fig. 4C). 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. 4D). Our results showed that LPA treatment did not alter the
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LPA<sub>2</sub> stimulation (Fig. 5A). Similarly, LPA stimulation promoted Siva-1 ubiquitination, and the levels of ubiquitinylated Siva-1 were further increased by MG-132 (Fig. 5B). Using MG-132 to prevent protein degradation, we found that the ubiquitinylated Siva-1 and LPA<sub>2</sub> receptor were present in the same complex, and this complex formation was increased by LPA treatment for 60 min (Fig. 5C). Together, these results suggest that prolonged LPA stimulation promotes the association of Siva-1 with the LPA<sub>2</sub> receptor and targets both proteins for proteasomal ubiquitination and degradation.

DNA Damage-induced Siva-1 Stabilization Is Attenuated by LPA in an LPA<sub>2</sub> 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. 6A). 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 pretreatment (Fig. 6B).

The counteracting role of LPA in regulating adriamycin-mediated Siva-1 stability was further demonstrated by assessing the turnover rate of transfected Siva-1 in NIH 3T3 cells treated with adriamycin and LPA (Fig. 6C). 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<sub>2</sub> receptor on the turnover of Siva-1 during DNA damage response. As shown in Fig. 6D, treatment with adriamycin and cycloheximide induced a robust activation and stabilization of Siva-1 in NIH 3T3 cells but not RH7777-LPA<sub>2</sub> cells, suggesting a role for LPA<sub>2</sub> 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<sub>2</sub> binding, overexpression of the LPA<sub>2</sub> receptor should not alter the expression of Siva-1<sup>ΔC3</sup> mutant that lacks the LPA<sub>2</sub> receptor-interacting domain (Fig. 1E). To test this expression of HA-Siva-1 in RH7777-mock cells but significantly attenuated its expression in RH7777-LPA<sub>2</sub> cells (Fig. 4D), suggesting that the LPA<sub>2</sub> receptor promotes LPA-induced Siva-1 degradation.

LPA Stimulation Increases Ubiquitination of Both Siva-1 Protein and the LPA<sub>2</sub> 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<sub>2</sub> receptor in response to LPA stimulation. To test this possibility, HEK 293T cells expressing HA-ubiquitin with FLAG-LPA<sub>2</sub> 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<sub>2</sub> receptor was found even in the absence of LPA (Fig. 5A). However, the levels of ubiquitinylated LPA<sub>2</sub> were increased by MG-132 pretreatment, which was further enhanced when followed by the presence of LPA (Fig. 5B). This result was further confirmed by overexpression of the Siva-1<sup>ΔC3</sup> mutant in NIH 3T3 cells (Fig. 5C). Together, these results suggest that prolonged LPA stimulation promotes the association of Siva-1 with the LPA<sub>2</sub> receptor and targets both proteins for proteasomal ubiquitination and degradation.

The levels of ubiquitinylated LPA<sub>2</sub> were increased by MG-132 (Fig. 5A). Similarly, LPA stimulation promoted Siva-1 ubiquitination, and the levels of ubiquitinylated Siva-1 were further increased by MG-132 (Fig. 5B). Using MG-132 to prevent protein degradation, we found that the ubiquitinylated Siva-1 and LPA<sub>2</sub> receptor were present in the same complex, and this complex formation was increased by LPA treatment for 60 min (Fig. 5C). Together, these results suggest that prolonged LPA stimulation promotes the association of Siva-1 with the LPA<sub>2</sub> receptor and targets both proteins for proteasomal ubiquitination and degradation.

FIGURE 4. LPA promotes the turnover of Siva-1 and the LPA<sub>2</sub> receptor. A, LPA promotes the turnover of the LPA<sub>2</sub> receptor. NIH 3T3 cells expressing FLAG-LPA<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> cells but not in RH7777-mock cells. HA-Siva-1 was expressed in RH7777-mock or RH7777-LPA<sub>2</sub> 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<sub>2</sub> and GAPDH in RH7777-mock and RH7777-LPA<sub>2</sub> 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.
**LPA<sub>2</sub> Mediates Siva-1 Degradation**

**FIGURE 5.** LPA induces ubiquitination of the LPA<sub>2</sub> receptor and Siva-1 and promotes the complex formation of ubiquitylated LPA<sub>2</sub> receptor and Siva-1. The HA-ubiquitin expression vector was co-transfected with the vector(s) expressing FLAG-LPA<sub>2</sub> receptor (A), FLAG-Siva-1 (B), Myc-Siva-1, and the FLAG-LPA<sub>2</sub> 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<sub>2</sub> 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 ubiquitylated LPA<sub>2</sub> receptor or Siva-1. The same blot was stripped and reprobed with an anti-FLAG polyclonal antibody to detect the FLAG-LPA<sub>2</sub> receptor (A) or FLAG-Siva-1 (B). Myc-Siva-1 co-immunoprecipitated with the FLAG-LPA<sub>2</sub> receptor was detected with an anti-Myc antibody (C).

**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<sub>2</sub> receptor but also concomitantly down-regulates LPA<sub>2</sub>-interacting Siva-1 protein. Consequently, the proapoptotic function of Siva-1 in DNA damage response is attenuated by LPA. Fig. 7D provides a model by which the interaction of Siva-1 with the LPA<sub>2</sub> 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<sub>1</sub> receptor is up-regulated, LPA protects cells from spontaneous apoptosis through an LPA<sub>1</sub>-dependent activation of AKT/PKB pathways (36). In contrast, in IEC-6 intestinal epithelial cells that express high levels of LPA<sub>2</sub> and lesser amounts of LPA<sub>1</sub>, an LPA<sub>2</sub>-selective agonist, FAP-12, protects cells from camptothecin-induced apoptosis (37). Moreover, another LPA<sub>2</sub> receptor-selective ago-
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 16 h 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 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. 1E. 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.

nist, 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 ubiquitination 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 LPA2 receptor but not other LPA receptors. However, there are some functional discrepancies between these two molecules in LPA signaling. TRIP6 specifically interacts with the LPA2 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 LPA2 receptor is not present (14). In contrast, Siva-1 binds to the LPA2 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 ubiquitinated LPA2 receptor and Siva-1 (Fig. 5 C). This result suggests that Siva-1 may preferentially bind to the internalized LPA2 receptor. The interaction of TRIP6 with the LPA2 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 LPA2 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 LPA2 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 LPA2 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 LPA2 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 LPA2 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.

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 LPA₂ antibody was a gift kindly provided by Dr. Anjaparavanda P. Naren. The immortalized double knock-out MEFs deficient in LPA1 and LPA2 were established by Drs. Natalia Makarova and Gabor Tigyi.

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