Lipid phosphatase-2 activity regulates S-phase entry of the cell cycle in Rat2 fibroblasts

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Lipid phosphatases are potent mediators of cell signaling and control processes including development, cell migration and division, blood vessel formation, wound repair, and tumor progression. Lipid phosphate phosphatases (LPPs) regulate the dephosphorylation of lipid phosphates, thus modulating their signals and producing new bioactive compounds both at the cell surface and in intracellular compartments. Knock-down of endogenous LPP2 in fibroblasts delayed cyclin A accumulation and entry into S-phase of the cell cycle. Conversely, overexpression of LPP2, but not a catalytically inactive mutant, caused premature S-phase entry, accompanied by premature cyclin A accumulation. At high passage, many LPP2 overexpressing cells arrested in G2/M and the rate of proliferation declined severely. This was accompanied by changes in proteins and lipids characteristic of senescence. Additionally, arrested LPP2 cells contained decreased lysophosphatidate concentrations and increased ceramide. These effects of LPP2 activity were not reproduced by overexpression or knock-down of LPP1 or LPP3. This work identifies a novel and specific role for LPP2 activity and bioactive lipids in regulating cell cycle progression.

The lipid phosphatases, lysophosphatidate (LPA)⁴ and sphingosine 1-phosphate (SIP)⁵ are present in biological fluids and activate cells through families of four G-protein-coupled receptors for LPA and five receptors for SIP (1). These receptors are coupled through G proteins that decreases cAMP concentrations; G₁₂/₁₃ that stimulates phospholipase C and increases actin stress fiber formation (16, 17). SIP also blocks activation of apoptosis in macrophages by inhibiting acidic sphingomyelinase activity (13).

Intracellular LPA can signal through the paxiosome proliferator-activated receptor-γ receptor (14) and sphingosine kinase-1, that regulates proinflammatory gene expression (15). Intracellular SIP stimulates ERK giving a mitogenic or anti-apoptotic response, it mobilizes intracellular Ca²⁺ and increases actin stress fiber formation (16, 17).

The lipid phosphate phosphatases (LPPs) are a family of enzymes that de-phosphorylate SIP, LPA, PA, and C1P, thus modulating their signaling (4). Such actions may also generate new signals through the dephosphorylated products sphingosine, diacylglycerol, and ceramide. There are three major isoforms of LPP, each containing six transmembrane spanning domains, an N-glycosylation site, which is not required for activity, and three conserved domains constituting a phosphatase active site (5). When the LPPs are expressed in the plasma membrane, the active site faces the extracellular matrix, thereby allowing LPPs to dephosphorylate external lipid phosphates. This orientation confers the potential to regulate the concentrations of extracellular LPA and SIP and possibly attenuate signaling through their respective receptors (18–20). Additionally, the extracellular activity of the LPPs promotes the uptake of dephosphorylated products of lipid phosphates, which has been shown to regulate cell movement and survival (21, 22). The LPPs are also expressed in intracellular membranes, and they can modify intracellular PA and DAG levels and perturb signaling downstream of G-protein-coupled receptors, including thrombin receptors (6, 23). Animal models have demonstrated that LPPs play important roles in regulating development, cell migration, tumor progression, and blood vessel formation (5, 22). Although each LPP isoform can have a distinct physiological impact, the specific target lipids and functions of the different isoforms are not well defined. LPP2 has a much more restricted distribution in organs than LPP1 and LPP3. LPP2 is therefore likely to have an isoform-specific biological function in tissues in which it is highly expressed compared with the other isoforms, such as in colon, pancreas, and ovary (24).

The present work arose from our observations that overexpressing LPP2 in fibroblasts produced a very different phenotype of cell proliferation compared with the overexpression of LPP1 or LPP3. Increasing LPP2 activity in rat2 fibroblasts caused a premature entry into S-phase associated with premature cyclin A expression. Conversely, knocking down endogenous LPP2 expression delayed S-phase entry associated...
with delayed cyclin A expression. The effects of LPP2 required its catalytic activity, and were not mimicked by increasing or decreasing LPP1 or LPP3 activity. Fibroblasts that stably overexpressed LPP2, but not LPP1 or LPP3, eventually arrested in G2/M after 20 passages and exhibited changes in the concentration of proteins and lipids that are characteristic of senescence. This work describes a novel, isoform-specific function of LPP2 that regulates cell cycle progression.

EXPERIMENTAL PROCEDURES

Cloning and Expression of LPPs—Rat2 cells and Bosc 31 packaging cells were described previously (18). cDNA for human LPP2, a gift from Dr. A. Morris (University of North Carolina, Chapel Hill, NC), or cDNA for rat LPP3 or mouse LPP1, were subcloned into the pBabePuro (pBP) expression vector. PCR was used to add a GFP tag to the C terminus of LPP2 and to create an R214K mutation. The pBP constructs were transiently transfected into retroviral Bosc 31 packaging cells and virion-containing media were used to infect rat2 fibroblasts. Mixed populations of transduced cells were selected by puromycin resistance (18). GFP-tagged human LPP2 and myc-tagged mouse LPP1 driven by a cytomegalovirus promoter were transferred into an adenovirus-packaging cell line using the AdEasy® vector system (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The recombinant plasmids were linearized and propagated in HEK 293 cells, and high-titer purified preparations (1 × 10^10 plaque-forming units/ml) were generated by the University of Iowa Gene Transfer Vector Core. For adenoviral transfections, multiplicity of infection 12 plaque-forming units/cell for myc-LPP1 or multiplicity of infection 100 plaque-forming units/cell for LPP2-GFP were added to cells in antibiotic-free media for 24 h.

Cell Culture—Rat2 fibroblasts were maintained in Dulbecco’s minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Medicorp Inc., Montreal, PQ, Canada) and an antibiotic/antimycotic mixture (penicillin/streptomycin/amphotericin B) (Invitrogen) at 5% CO_2, 95% humidity, and 37 °C.

Immunofluorescence Microscopy—Cells were plated on coverslips coated with fibronectin (Sigma). Cells were fixed in buffered 4% formaldehyde, permeabilized, blocked in 4% nonfat milk and 0.6% bovine serum albumin, and incubated with primary and fluorescence-conjugated secondary antibodies (25). Coverslips were mounted with ProLong® antifade mounting media (Invitrogen). Fluorescence was viewed on a Zeiss 510 confocal microscope using a pinhole of 1 airy unit and co-localization was determined using LSM5 Software (Carl Zeiss Inc.). Goat polyclonal anti-GFP from Dr. L. Berthiaume (University of Alberta, Edmonton, AB, Canada) was diluted to 1:200. Mouse anti-early endosome antigen-1 from BD Biosciences (E41120) was diluted 1:200. Rabbit anti-caveolin-1 from Upstate Biotechnology (Charlottesville, VA) (06-591) was diluted 1:100. Secondary chicken anti-rabbit Alexa Fluor®594 (A-21442), chicken anti-mouse Alexa Fluor 594 (A-21201), and chicken anti-goat Alexa Fluor 488(A-21467) from Invitrogen were diluted 1:500.

siRNA Transfection—Double-stranded SMARTpool® siRNAs targeting rat LPP1, rat LPP2, rat LPP3, cyclophilin B, and non-targeting controls were purchased from Dharmacon (Lafayette, CO). Lipofoctamite 2000 (Invitrogen) in Opti-MEM (Invitrogen) was used at 0.625 μg/ml according to the manufacturer’s protocol. The final concentration of siRNAs was 200 nM. Controls for the knock-downs were performed with cyclophilin B, non-targeting control siRNAs, and lipofoctamite alone. It was determined experimentally that maximum knock-down was achieved at and remained constant between 40 and 72 h post-transfection. The transfection efficiency for the introduction of siRNA was about 90%, as evaluated by the number of fluorescent cells transfected with siGLO, divided by the number of nuclei stained with Hoechst 33258 or phase-contrast microscopy (results not shown). For cell cycle analysis, transfection was performed in antibiotic-free media containing serum, and media were changed 6 h after transfection. After a further 18 h of transfection, cells were treated with serum-free media for 20 h before the re-addition of serum to promote cell cycle progression. Lysates were collected for real time RT-PCR at 12 h after the addition of serum in each experiment to determine the extent of knock-down achieved at approximately the point of S-phase entry.

Real-time RT-PCR—RNA was collected using the RNAqueous kit (Ambion Inc., Austin, TX) according to the manufacturer’s directions. Contaminating DNA was removed using the DNA-free kit (Ambion) according to the manufacturer’s directions. RNA was quantitated spectrophotometrically at 260 nm. Reverse transcription was performed using Superscript II (Invitrogen), random primers (Invitrogen), and RNAout (Invitrogen) according to the manufacturer’s instructions. Negative controls lacking RNA or RT were performed with each reverse transcription reaction. PCR was performed on an iCycler (Bio-Rad). Each reaction contained 0.2 μM of each primer, ~100 ng of cDNA from the reverse transcription reaction, and SYBR Green® PCR master mixture (Applied Biosystems, Foster City, CA). Standard curves were generated for each primer pair and the slope and efficiency calculated from the curves were used to determine target RNA levels relative to the housekeeping gene cyclophilin A. Melting curves were performed with each analysis to determine product specificity, and amplified products were run out in 2% agarose to confirm the presence of a single band. An annealing temperature of 57 °C was used for all primer pairs. Primers for PCR were as follows: LPP2 forward, TGGCCAAGTAGT-CATGATTGG and reverse, AGCAGCGCGTGCCACCTTCC; LPP1 forward, GTGTCAAAAATCAACTGCG and reverse, TGGCTGTGAG-ATAAAGTGC; LPP3 forward, CCCCCGCCTCAACAAACC and reverse, TCTCTGATGATGAGGAAGGG; and mouse cyclophilin A forward, CACCGTGTTCTTCGACATCAC and reverse, CCAGTGCTCA-GAGCTCGAAAG. Primers for the LPPs were designed to recognize human, mouse, and rat sequences.

Measurement of Lipid Phosphatase Activity—Lysates were collected in 1% Nonidet P-40, 10% glycerol, 50 mM HEPES, 137 mM NaCl, 1 mM MgCl_2, 1 mM CaCl_2, 10 mM Na_2PO_4, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. Samples were assayed for protein with a bicinechonic acid assay (Bio-Rad), and for the formation of diacylglycerol from 3H-labeled S1P (17). For measurements of evo-activity, medium containing 10 μM [32P]SIP or 5 μM [32P]S1P was added for 30 min. Media were collected and 32P was extracted (17).

Analyses of Proliferation and Apoptosis—Cells were seeded at 30,000 cells/dish and grown for 8 days, with fresh media added each day. Under these conditions, cells proliferated exponentially for 2–3 days before encountering contact inhibition, irrespective of passage number. Cells were washed with HEPES-buffered saline, trypsinized, resuspended in growth media, and counted on a hemocytometer. Parallel determinations of protein and DNA content were performed in some cases using the bicinechonic acid assay (Bio-Rad) and Hoechst staining in a 96-well plate (26), respectively. For measurement of apoptosis, cells were fixed with buffered 4% formaldehyde and stained with 500 ng/ml Hoechst 33258. Apoptotic cells were quantitated by counting condensed and/or fragmented nuclei versus evenly stained nuclei (27).

Cell Cycle Analysis—Cells were synchronized by starvation in Dulbecco’s minimum essential medium containing 0.68% fatty acid-free bovine serum albumin (Sigma) and released after 24 h by adding Dulbecco’s minimum essential medium containing 10% FBS. Cells synchronized by trypsinization exhibited the same phenotype (results not shown). Nocodazole and double thymidine block techniques were not
Results are mean ± S.D. from at least four independent experiments. Statistically significant differences (p < 0.05) from control are indicated by the asterisk.
LPP2 Regulates S-phase Entry

The overexpression of LPP2, rat2 fibroblasts were transduced with hLPP2, hLPP2-GFP, mutant LPP2(R214K)-GFP, mLPP1, mLPP1-GFP, mLPP3-GFP, or myc-rLPP3 and stable cell populations were selected with puromycin without clonal selection. Cells transduced with LPP2, LPP2-GFP, and R214K-GFP showed 32-, 42-, and 28-fold increases in mRNA for LPP2, respectively, compared with the endogenous expression of LPP2. The overexpression of each of the three LPPs did not significantly alter the expression of mRNA for the other isoforms (Fig. 1, A and B). Transfection with control siRNAs did not decrease the expression of any LPP isoforms, and the knock-down of each of the three LPPs did not significantly alter the expression of mRNA for the other isoforms (Fig. 1, A and B).

To overexpress the LPPs, rat2 fibroblasts were transduced with hLPP2, hLPP2-GFP, mutant LPP2(R214K)-GFP, mLPP1, mLPP1-GFP, mLPP3-GFP, or myc-rLPP3 and stable cell populations were selected with puromycin without clonal selection. Cells transduced with LPP2, LPP2-GFP, and R214K-GFP showed 32-, 42-, and 28-fold increases in mRNA for LPP2, respectively, compared with the endogenous expression levels in cells transduced with empty vector (Fig. 1C). Overexpression of LPP1 and LPP3 resulted in 16- and 78-fold increases in mRNA for the targeted LPP (Fig. 1, A and B). Transfection with control siRNAs did not decrease the expression of any LPP isoforms, and the knock-down of each of the three LPPs did not significantly alter the expression of mRNA for the other isoforms (Fig. 1, A and B).

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RESULTS

Characterization of Fibroblasts with Modified Expression of the LPPs—To study the isoform-specific effects of the LPPs, techniques for decreasing and increasing the relative expression of the three isoforms were developed. Knock-down experiments were performed by transfecting cells with siRNAs for each of the rat LPP isoforms. Real-time RT-PCR demonstrated that rat2 fibroblasts treated with siRNAs for LPP1, LPP2, and LPP3 showed about a 60% decrease in mRNA for the targeted LPP (Fig. 1, A and B). Transfection with control siRNAs did not decrease the expression of any LPP isoforms, and the knock-down of each of the three LPPs did not significantly alter the expression of mRNA for the other isoforms (Fig. 1, A and B).

LPP2 protein levels could not be determined because of technical difficulties encountered in resolving the protein on SDS-PAGE. Various techniques, which allowed the resolution of LPP1-GFP and LPP3-GFP with anti-GFP, including the addition of urea, increased detergent concentrations, N-ethylmaleimide addition, and lack of boiling, all failed to resolve LPP2-GFP and untagged LPP2 using two different anti-LPP2 antibodies (23, 32) or an anti-GFP antibody. Immunoprecipitation of LPP2-GFP with anti-GFP antibody demonstrated that recombinant LPP2 activity was recovered (Fig. 2A), and there was no soluble GFP detected on Western blots (results not shown). This indicated that the LPP2-GFP fusion protein was overexpressed and remained intact. The immunoprecipitate from cells stably overexpressing LPP2-GFP did not co-immunoprecipitate LPP1, even when cells were transfected with adenovirus expressing myc-mLPP1 to optimize any possible interaction between LPP1 and LPP2. Immunoprecipitations were performed with anti-GFP, and lysates and immunoprecipitates were resolved on SDS-PAGE. Western blots were performed on the same membrane using mouse anti-myc (upper panel) and rabbit anti-mLPP1 (lower panel). Membranes were scanned simultaneously with an Odyssey® imager, at 700 and 800 nm to detect the two secondary antibodies, respectively. Panel C shows LPP activities in cells treated with siRNA for non-targeting control or for rat LPP1, LPP2, or LPP3. Panel D shows LPP activities of stable cell lines that express empty vector (pBP), LPP2, LPP2-GFP, R214K-GFP, LPP1, and LPP3. Results are expressed as fold change compared with rat2 fibroblasts (92), which is given as 1. Results are mean ± S.D. from at least four independent experiments. Statistically significant differences (p < 0.05) from control are indicated by the asterisk.

FIGURE 2. Activity of the LPP2-GFP recombinant protein and total lipid phosphatase activity in cells with altered LPP expression. Panel A shows the LPP activity of anti-GFP immunoprecipitates that were obtained by treating the indicated amount of lysate protein from cells stably overexpressing GFP (●) or LPP2-GFP (●). Results are mean ± S.D. from at least four independent experiments. In Panel B, fibroblasts stably overexpressing LPP2-GFP were transfected with adenovirus for myc-mLPP1 to optimize any possible interaction between LPP1 and LPP2. Immunoprecipitations were performed with anti-GFP, and lysates and immunoprecipitates were resolved on SDS-PAGE. Western blots were performed on the same membrane using mouse anti-myc (upper panel) and rabbit anti-mLPP1 (lower panel). Membranes were scanned simultaneously with an Odyssey® imager, at 700 and 800 nm to detect the two secondary antibodies, respectively. Panel C shows LPP activities in cells treated with siRNA for non-targeting control or for rat LPP1, LPP2, or LPP3. Panel D shows LPP activities of stable cell lines that express empty vector (pBP), LPP2, LPP2-GFP, R214K-GFP, LPP1, and LPP3. Results are expressed as fold change compared with rat2 fibroblasts (92), which is given as 1. Results are mean ± S.D. from at least four independent experiments. Statistically significant differences (p < 0.05) from control are indicated by the asterisk.
Lipid Phosphatase Activity in Transfected Cells—Total lipid phosphatase activity consists of the combined activities of the three LPP isoforms and it was measured in whole cell lysates using PA in Triton X-100 micelles. In cells in which endogenous LPP2 expression was knocked down by 61%, there was no change in total LPP activity (Fig. 2C). However, we are confident that this level of down-regulation of LPP2 mRNA is biologically relevant because it resulted in a clear phenotype (see next section). Knock-down of LPP3 also failed to significantly change lipid phosphatase activity, however, knock-down of LPP1 to 48% of endogenous levels produced a 53% decrease in total LPP activity (Fig. 2C). This suggests that LPP1 is the major contributor to endogenous LPP activity in the fibroblasts. Furthermore, knock-down of LPP1 may be expected to change bulk lipid concentrations in the cells, whereas knock-down of LPP2 or LPP3 would be less likely to do so.

Cells overexpressing untagged and GFP-tagged LPP2 exhibited 2.9- and 2.2-fold increases in total LPP activity, respectively, compared with the vector control (Fig. 2D). Cells overexpressing LPP2(R214K)-GFP showed no significant change in LPP activity, as expected for an inactive mutant (Fig. 2D). Cells overexpressing LPP1 and LPP3 had increased LPP activity by 3.9- and 2.2-fold, respectively (Fig. 2D).

Ecto-LPP activity was measured in intact cells as the dephosphorylation of 10 μM LPA or 5 μM S1P in the extracellular medium. The overexpression of LPP2 did not significantly change the hydrolysis of extracellular LPA or S1P (results not shown). The overexpression of LPP1 and LPP3 did increase the hydrolysis of extracellular LPA and S1P (results not shown).

Localization of LPP2 in Rat2 Fibroblasts—Confocal studies were performed using antibodies to the GFP tag on LPP2 and to various organelle markers. Wild-type and mutant LPP2 showed the same localization profile, which differed from the ubiquitous cellular distribution of GFP alone (supplementary Fig. i). LPP2-GFP and LPP2(R214K)-GFP were localized to the plasma membrane and intracellular membranes. Co-localization studies indicated that LPP2 was found in the early endosomes co-localized with early endosome antigen-1, and co-localized with caveolin-1 at the plasma membrane and in intracellular membranes (supplementary Fig. i). Partial co-localization was observed with the endoplasmic reticulum marker calnexin (results not shown). LPP2 did not co-localize significantly with markers for the Golgi apparatus, mitochondria, nucleus, or nuclear membrane (results not shown). The likely sites of action for LPP2, therefore, include the plasma membrane, endosomes, and endoplasmic reticulum, and it is unlikely that LPP2 acts in the nucleus. Importantly, these results demonstrate that the mutant LPP2 is not mislocalized, and validate using the mutant to distinguish the catalytic versus non-catalytic functions of LPP2.

Decreasing LPP2 Expression Delays S-phase Entry whereas Increasing LPP2 Activity Causes Premature Entry into S-phase—To examine the role of endogenous LPP2 in controlling S-phase entry, we knocked down LPP2 mRNA in rat fibroblasts by an average of 61% in three experiments. We were unable to measure if there was a proportional decrease in LPP2 protein because of the problems described above for Western blotting and lack of antibodies that could detect the low endogenous levels of untagged LPP2. Cells transfected with control siRNAs or
**LPP2 Regulates S-phase Entry**

**FIGURE 5.** Prolonged LPP2 overexpression causes a decreased rate of cell proliferation and increased accumulation in G2-phase. Panel A shows the rate of proliferation of cells stably expressing empty vector ( ), LPP2 ( ), or LPP2(R214K) ( ). Panel B shows the number of cells after 8 days of growth in fibroblasts stably overexpressing GFP-tagged LPPs, or GFP alone. Panel C shows cell cycle distribution after 10 days of growth of stable cells transfected with empty vector or LPP2 and unsynchronized. Results are one representative of three independent experiments.

rat LPP2 siRNAs were synchronized, and their progression through the cell cycle was measured by flow cytometry. Decreasing endogenous LPP2 expression delayed entry into S-phase by 1.2 ± 0.14 h (mean ± S.D. for three independent experiments) compared with parental control cells, or cells transfected with non-targeting control siRNAs (Fig. 3A). These results confirm that the LPP2 mRNA knock-down produced a physiologically important decrease in LPP2 activity. Furthermore, the effect was specific, because decreasing endogenous LPP1 or LPP3 did not alter the rate of S-phase entry (Fig. 3B). Conversely, LPP2 overexpressing cells entered S-phase 2.4 ± 0.70 h (6 experiments) before control fibroblasts that expressed cDNA for the empty vector, or than those expressing LPP2(R214K) (Fig. 3C). Fibroblasts that overexpressed LPP1 and LPP3 entered S-phase at approximately the same time as vector control cells (Fig. 3D).

**Increasing LPP2 Activity Causes Premature Cyclin A Expression and Decreased LPP2 Expression Delays Cyclin A Expression**—Levels of the cyclins that regulate cell cycle progression into S-phase were measured at different times to determine the mechanism of the early S-phase entry. Western blots were quantitated and the results were presented as relative expression levels. These values can be compared within, but not across experiments. Decreasing endogenous LPP2 mRNA delayed cyclin A expression compared with cells treated with control siRNAs (Fig. 4A). Consequently, decreased cyclin A expression occurred between 8 and 14 h after the addition of FBS, prior to S-phase entry. In cells overexpressing catalytically active LPP2, cyclin A expression was accelerated by about 2 h (Fig. 4B). This 2-h acceleration paralleled the 2-h acceleration in S-phase entry. Cells overexpressing LPP1 or LPP3 were indistinguishable from vector control cells in terms of both the timing and magnitude of expression of cyclin A (Fig. 4C). Overexpression of the inactive mutant LPP2(R214K)-GFP did not accelerate the expression of cyclin A (results not shown). LPP2 overexpression did not change the magnitude or timing of expression of cyclins D1, D2, D3, or E, cyclin-dependent kinase-2, Ser\(^{\text{P}}\)-phosphorylated p53, p21\(^{\text{CIP1}}\), or p27 (results not shown). Therefore it is probable that LPP2 controls S-phase entry by regulating the timing of cyclin A expression.

**Cells Transduced with LPP2 Show Decreased Rates of Proliferation at High Passage and Accumulate in G2/M—**During our work in culturing cells that overexpressed different LPPs, we consistently observed that the LPP2 overexpressing fibroblasts progressively slowed in their proliferation rates. Cells at passage 24 were seeded at low density and their proliferation was measured for 8 days. After 8 days of growth, control cells and cells overexpressing LPP2(R214K) had increased in number by ~40-fold, whereas the numbers of LPP2-transduced cells had increased by only 5-fold (Fig. 5A). Cells transduced with LPP1 or LPP3 proliferated to the same extent as control cells of the same passage (Fig. 5B). The addition of up to 30% fetal bovine serum, 50 μM LPA, or 5 μM S1P to the media did not overcome the decrease in proliferation exhibited by LPP2-transduced cells (results not shown). The decreased proliferation of LPP2-transduced cells was not caused by increased apoptosis, because both control and LPP2-transduced cells contained only about 1% apoptotic cells, as determined by Hoechst staining or by measuring the subdiploid peak in flow cytometry (results not shown).

To understand the decreased proliferation rate of the LPP2-transduced fibroblasts, we investigated cell cycle progression. After 15–20 passages, cells transduced with LPP2 began to accumulate in G2/M. Confluent parental rat2 and vector control fibroblasts at passage 24 contained 85–90% of the cells in G2 phase and only 4% in G2/M, as expected (Fig. 5C). Cells transduced with LPP1, or LPP3, or with inactive mutant LPP2 also contained over 80% of cells in G2-phase and less than 8% of cells in G2/M-phases at confluence (results not shown). By contrast, at passage 24, about 29% of the cells that were transduced with catalytically active LPP2 were in G2/M-phase (Fig. 5C). This number increased with increasing passage number, reaching 70% of cells by passage 35 (results not shown). Hoechst staining confirmed a proportional increase in DNA content per cell in the LPP2-transduced, G2-arrested cells (results not shown). At passage 35, cell proliferation became...
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Cells Transduced with LPP2 Show Characteristics of Senescence at High Passage—LPP2-transduced cell populations at late passage number, in which greater than 30% of cells were arrested in G2, displayed many changes in protein expression that are characteristic of DNA damage or senescence. The level of phospho-p53 (Ser15) was elevated 16-fold, and expression of p21(WAF1), p27, and p16 were increased by 8-, 6-, and 7-fold, respectively (Fig. 6E). Additionally, cyclins D1, D2, D3, and E were increased 5-, 7-, 2-, and 4-fold, respectively (Fig. 6E). These increases in cyclin expression are consistent with previous studies in which cyclin D and E levels were elevated in senescent cells (35, 36). Surprisingly, LPP2-transduced cells containing more than 50% of cells in G2/M with an activated G2/M checkpoint also eliminated the overexpression of LPP2, as determined by real-time RT-PCR (results not shown). At passage 35, LPP2 mRNA levels were not statistically different from LPP2 mRNA expression levels in rat2 control cells.

Cells arrested in G2 were also analyzed for lipid content. G2/M-arrested cells contained more than twice the relative amount of ceramide of parental cells (Table 1). G2-arrested cells also showed a 50% decrease in LPA levels relative to total phospholipid (Table 1). Sphinganine phosphate levels also appeared to have increased in G2-arrested cells, but the effect was not statistically significant. The changes observed in ceramide and LPA concentrations were not observed in cycling LPP2-overexpressing cells at early passages, and are therefore related to the G2/M arrest phenotype. Other lipids measured including ceramide 1-phosphate, sphingosine, sphingosine 1-phosphate, and sphinganine were not changed significantly in G2-arrested cells compared with control cells (Table 1). Phosphatidate and total and nuclear diacylglycerol levels were also not significantly different in LPP2-overexpressing or in G2-arrested cells compared with control fibroblasts (results not shown).

DISCUSSION

Little is known about the specificity and functions of the different LPP isoforms and how they differentially modify cell signaling. In this study we demonstrate that LPP2 regulates cell cycle progression. Decreasing the expression of endogenous LPP2 delays S-phase entry, whereas increasing LPP2 expression results in premature entry into S-phase. LPP2 catalytic activity was required for these effects because expression of the inactive LPP2(R214K) mutant did not change the rate of S-phase entry. LPP2 catalytic activity was required for these effects because expression of the inactive LPP2(R214K) mutant did not change the rate of S-phase entry.
entry. The use of the inactive mutant is justified because the mRNA expression and subcellular distribution of the protein were not significantly different from wild-type LPP2. The effects of LPP2 were isoform specific, because increasing or decreasing the expression of LPP1 or LPP3 using the same protocols did not alter the rate of S-phase entry. The profound effects produced by knocking down endogenous LPP2 activity illustrates that LPP2 is an important regulator of S-phase entry. This work, therefore, provides the first evidence of an isoform-specific biological function for LPP2 activity in regulating cell cycle progression.

Overexpression of catalytically active LPP2 resulted in premature S-phase entry after synchronization by serum deprivation. We ensured that this did not result from inadequate arrest in G2 during serum deprivation (see "Experimental Procedures"). To ensure that overexpression of LPP2 reproducibly and selectively accelerated S-phase entry, we transduced rat2 fibroblasts by retroviral infection with human LPP2 or human LPP2 tagged at the C terminus with GFP. Polyclonal cell populations were used and LPP2 was subcloned into both the pBabePuro and pLNCX2 vectors, which have different selection markers. Stable cell populations transduced with empty vector, untagged LPP2, LPP2-GFP, LPP2(R214K)-GFP, LPP1, and LPP3 were created on four separate occasions. Every cell population created that overexpressed catalytically active tagged or untagged LPP2 entered S-phase prematurely. These results establish that the GFP tag on LPP2 did not change its effect on cell cycle regulation. By contrast, every cell population expressing the empty vector control, LPP1, LPP3, or mutant LPP2(R214K) did not show changes in the timing of entry into S-phase.

The effect of LPP2 on S-phase entry appears to be regulated through cyclin A. The increase in cyclin A expression was accelerated by about 2 h in cells overexpressing LPP2 activity and it was delayed by about 1.5 h in cells with decreased LPP2 expression. These changes corresponded to the acceleration, or delay in S-phase entry. The changes in cyclin A also required the catalytic activity of LPP2, and cyclin A expression was not changed by modulating the activities of LPP1 or LPP3. Cyclin A is a partner of cyclin-dependent kinase-2 (CDK2), which regulates G1 to S-phase progression. Dysregulation of cyclin A expression and subsequent increases in cyclin A-associated CDK2 activity leads to unscheduled progression into S-phase (37–44). The expression of other cell cycle regulatory proteins (p21 and p27, cyclins D1, D2, D3, and E) were unchanged in LPP2 overexpressing cells that entered S-phase prematurely. Furthermore, differences in cyclin A expression occurred at time points prior to S-phase entry. We, therefore, conclude that LPP2 mediates its effects on S-phase entry primarily through regulating the timing of cyclin A expression. Several kinases that influence cyclin A expression and G1 to S-phase progression (ERK, p38 MAPK, Akt, and LIMK) were not changed in expression level, timing of expression, or phosphorylation state in cells that overexpressed LPP2 and entered S-phase prematurely (results not shown). We, therefore, conclude that LPP2 does not increase cyclin A expression through ERK, p38 MAPK, Akt, or LIMK. To determine whether LPP2 expression is itself regulated during cell cycle progression, we measured endogenous levels of LPP2 mRNA in rat2 fibroblasts during starvation and throughout the 24 h of the cell cycle following stimulation with serum. The level of mRNA for LPP2 remained constant during starvation and during cell cycle progression (results not shown). These results do not exclude the regulation of LPP2 activity by post-translational modification or subcellular localization to control the rate of S-phase entry in relevant physiological situations.

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### TABLE 1

Lipid composition of G2 arrested cells

| Cells                        | Relative concentration | Lyso phosphatidic acid |
|------------------------------|------------------------|------------------------|
|                              | Ceramide               | Ceramide-P             | Sphingosine | Sphingosine-P | Sphinganine | Sphinganine-P | -Fold increase |
| R2                           | 3.81 ± 0.31            | 79.1 ± 5.47            | 0.333 ± 0.001| 1.27 ± 0.201 | 0.533 ± 0.067| 0.071 ± 0.021| 1.00 ± 0.000  |
| LPP2 (arrested in G2)        | 6.71 ± 0.47*           | 76.8 ± 2.19            | 0.429 ± 0.138| 1.16 ± 0.273 | 0.676 ± 0.141| 0.127 ± 0.031| 0.0368 ± 0.052*|
| Vector control               |                        |                       |             |              |             |              | 1.00 ± 0.000  |
| LPP2 (low passage)           |                        |                       |             |              |             |              | 1.07 ± 0.102  |

* Statistical significance (p < 0.05) from rat2 fibroblasts or vector control.
had markedly increased levels of cyclins D1, D2, D3, and E, phosphorylated p53 (Ser15), p21, p27, and p16INK4a, characteristic of a G2-arrested or senescent phenotype (35, 36, 46, 47). In these cell populations, cycling was virtually undetectable and cyclin levels did not vary over time, even after cells were starved by serum deprivation. Cyclin B levels were reduced compared with unsynchronized control cells, and cyclin A levels were similar to the levels in control cells. The level of inhibitory phosphorylation of Tyr15 on CDK1 was increased in G2-arrested cells similar to that at its maximal activation prior to the G2/M transition in cycling control cells, and remained constitutively at this level. Increased Tyr15 phosphorylation of CDK1 is commonly observed in cells with DNA damage. The G2/M checkpoint activation in late passage cells transduced with LPP2 likely resulted from accumulation of DNA damage resulting from repeated premature S-phase entry because LPP2 overexpressing cells at low passage showed normal expression of cyclin B and normal regulation of CDK1 phosphorylation. In cultured cells, some oncogenes can induce premature senescence after initially stimulating proliferation, and this process may represent a physiological response involved in preventing malignancy (48, 49). This type of senescence is characterized by the up-regulation of p53 and p16INK4a (48).

Fibroblast populations that were largely arrested in G2, as a consequence of initial LPP2 overexpression contained about twice as much ceramide as control cells. Different ceramide species were increased proportionally, and the predominant species, 16:0, comprised 50% of the total ceramide. Ceramide levels increase in senescent cells and proportionally, and the predominant species, 16:0, comprised 50% of ceramide as control cells. Different ceramide species were increased sequent to initial LPP2 overexpression contained about twice as much LPA is an agonist for the peroxisome proliferator-activated receptor (14), which decreases the synthesis of several proteins that are phosphorylated in G2-arrested cells. These levels were similar to the levels in control cells. The level of inhibitory phosphorylation of Tyr15 on CDK1 is commonly observed in cells with DNA damage. The G2/M checkpoint activation in late passage cells transduced with LPP2 likely resulted from accumulation of DNA damage resulting from repeated premature S-phase entry because LPP2 overexpressing cells at low passage showed normal expression of cyclin B and normal regulation of CDK1 phosphorylation. In cultured cells, some oncogenes can induce premature senescence after initially stimulating proliferation, and this process may represent a physiological response involved in preventing malignancy (48, 49). This type of senescence is characterized by the up-regulation of p53 and p16INK4a (48).

Our results indicate that LPP2 regulates timing of entry into S-phase, but it is not essential for cell-cycle progression. Several genes that regulate progression into late G1 or entry into S-phase have been knocked out in mice without lethality or other major generalized phenotypes. These knockouts include critical cell-cycle regulators such as CDK2, CDK4, CDK6, and cyclins D1, D2, D3, E1, or E2 (reviewed in Ref. 53). Therefore, deletion of LPP2 would not be expected to result in lethality or any other major generalized phenotype. Consistent with this expectation, LPP2 knock-out mice are viable and overtly normal (54). By contrast, knocking out LPP3 expression causes embryonic lethality (55). Transgenic mice that overexpress LPP1 have decreased birth weight, sparse curly hair, and defective spermatogenesis causing infertility (45). Therefore, these studies with mouse models support our work demonstrating that LPP2 has a unique and isoform-specific function that is not exhibited by LPP1 and LPP3. Our studies show that this unique function is the regulation of the timing of entry into S-phase.

In summary, this study demonstrates that LPP2 is a regulator of cell cycle progression in fibroblasts. Decreasing the expression of LPP2 caused a 1.5-h delay in entry into S-phase following the delayed expression of cyclin A. Overexpression of LPP2 caused the premature expression of cyclin A and a 2-h premature entry into S-phase. These represent substantial changes in the rate of S-phase entry that could have implications in processes such as mitogenesis, migration, wound healing, development, and tumorigenesis. Cell cycle regulation depended on the catalytic activity of LPP2, and this effect was isoform specific. Overexpression or knock-down of LPP1 or LPP3 did not alter S-phase entry. Cells that overexpressed catalytically active LPP2, but not inactive LPP2, LPP1, or LPP3, accumulated in G2/M-phase of the cell cycle progressively after 20 passages as a result of activating the G2/M checkpoint. These cells eventually stopped proliferating and exhibited changes in protein and lipid concentrations characteristic of DNA damage and senescence. This work provides the first evidence of a catalytic and isoform-specific function of LPP2 as a cell cycle regulator.

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