StARD13(Dlc-2) RhoGap Mediates Ceramide Activation of Phosphatidylglycerolphosphate Synthase and Drug Response in Chinese Hamster Ovary Cells

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To identify genes involved in etoposide drug response, we used promoter trap mutagenesis to isolate an etoposide-resistant Chinese hamster ovary (CHO) cell line. This resistant CHO-K1 line, named E91, showed cross-resistance to C2-ceramide (N-acetylphosphosine). The promoter trap retrovirus was found integrated into intron 1–2 of the Dlc-2 (Stard13) RhoGap gene. The E91 cells showed elevated guanosine triphosphate (GTP)-bound RhoA levels compared with the parental line, suggesting that retrovirus integration had inactivated one of the Dlc-2 RhoGap alleles. To test whether E91 cells were impaired in an intracellular ceramide-regulated process not directly related to cell killing, we measured mitochondrial phosphatidylglycerolphosphate (PGP) synthase and phospholipase A2 enzyme activities in cells after C2-ceramide addition. Parental cells showed elevated enzyme activities after treatment with C2-ceramide or tumor necrosis factor α, but not the E91 cells. These results suggested that intracellular ceramide signaling was defective in E91 cells due to increased levels of active GTP-bound RhoA. RNA knockdown experiments of the Dlc2 RhoGap resulted in increased GTP-bound RhoA and reduced induction of PGP synthase after C2-ceramide addition compared with controls. Expression of a dominant-negative RhoA in the E91 cell line allowed induction of PGP synthase by ceramide. The RNA interference knockdown cell line also showed increased etoposide resistance. This study is the first report for the regulation of a phospholipid biosynthetic enzyme through RhoGap expression.

INTRODUCTION
Ceramides are involved in the regulation of cellular growth, differentiation, and apoptosis (for reviews, see Spiegel and Merrill, 1996; Gomez-Munoz, 1998; Mathias et al., 1998). Ceramides are generated de novo or from the hydrolysis of sphingomyelin (SM) on the outer leaflet of the plasma membrane and from intracellular SM in many different cell types. Recently, the use of short chain cell-permeable ceramides (e.g., C2-Cer) has greatly facilitated research in ceramide-induced cell signaling processes. These short chain cell-permeable ceramides rapidly enter into the cell, and they mimic the generation of intracellular ceramide. Several studies support the existence of a signal-transducing pool of SM that is distinct from the pool of SM accessible to exogenous SMase (for review, see Gomez-Munoz, 1998). Incubation with bacterial SMase, which hydrolyzes SM on the outer leaflet of the plasma membrane, produces an extracellularly derived pool of ceramide for cell signaling. The use of cell-permeable ceramides has enabled researchers to distinguish the effect of intracellularly derived ceramides on metabolic processes from ceramide generated at the external surface of the plasma membrane. For example, in U987 cells the biological actions of ceramide were shown to depend upon its subcellular topology of production (Wiegmann et al., 1994). Ceramide generated from plasma membrane-associated neutral SMase-activated pathways are independent of ceramide generated from endosomal acidic SMase. When Molt-4 leukemia cells were transfected with Bacillus cereus SMase, the resultant increase in SMase activity and intracellular levels of ceramide resulted in cleavage of poly(ADP-ribose) polymerase and hence apoptosis (Zhang et al., 1997). In contrast, addition of exogenous B. cereus SMase to these cells did not induce apoptosis. Tumor necrosis factor (TNF) α binding to its receptor activates both a membrane-associated neutral SMase, and an endosomal acidic-SMase resulting in distinct subcellular topology of intracellular ceramide production (Wiegmann et al., 1994).

Some chemotherapeutic drugs and radiation can increase endogenous ceramide levels in cells, and this may play a role in cell killing. Daunorubicin can increase ceramide levels through stimulation of the SMase activity in the cell (Jaffrezou et al., 1996) or an increase in ceramide synthase activity (Bose et al., 1995). Mouse and human cells deficient in acid SMase showed resistance to radiation induced apo...
ptosis (Santana et al., 1996). The topoisomerase inhibitor etoposide can also increase de novo ceramide synthesis through the activation of serine palmitoyltransferase activity (Perry et al., 2000) and sphingomyelin hydrolysis by neutral SМase induction (Sumitomo et al., 2002).

Cardiolipin (CL) is an important structural and functional phospholipid in eukaryotic and prokaryotic cells (for reviews, see Hostetler, 1982; Hatch, 2004). The committed step in the biosynthesis of CL involves the conversion of cytidine-5'-diphosphate-1,2-diacylglycerol (CDP-DG) and sn-glycerol-3-phosphate to phosphatidylglycerol (PGP) in a reaction catalyzed by PGP synthase (EC 2.7.8.5). The PGP synthase gene has been identified in Saccharomyces cerevisiae (Chang et al., 1998), and the enzyme has also been purified from Schizosaccharomyces pombe (Jiang et al., 1998). A mammalian temperature-sensitive Chinese hamster ovary (CHO) cell mutant with a thermolabile PGP synthase had been isolated previously (Ohtsuka et al., 1993). This mutant was defective in both phosphatidylglycerol (PG) and CL production when grown at the nonpermissive temperature. These mitochondrial abnormalities were corrected by expression of the cDNA for PGP synthase in the temperature-sensitive CHO mutant cells (Kawasaki et al., 1999). CL levels can also play an important role in targeting of the cleaved proapoptotic protein Bid (tBid) to mitochondrial membranes (Lutter et al., 2000).

Little information concerning the signaling processes involved in the regulation of PGP synthase in mammalian cells is currently available. Previously, we demonstrated that PGP synthase activity in H9c2 cardiac myoblast cells could be regulated by ceramide signaling (Xu et al., 1999). Addition of exogenous C2-Cer or TNFα-α-mediated generation of endogenous ceramide resulted in elevation of mitochondrial PGP synthase activity. Transfection of cells with bacterial SMase or additions of cell-permeable ceramides are useful means to elevate intracellular ceramide levels. However, one problem in the study of ceramide signaling has been the lack of cell mutants in which the generated ceramide signal could be blocked or attenuated. If such ceramide-signaling mutants existed then one could probe for the effect of ceramide signaling on various ceramide-regulated cellular processes. In a study to isolate etoposide-resistant mutants by using a promoter trap retrovirus, we found one cell line that showed cross resistance to C2-Cer. In the present study, we show that this mutant CHO cell line shows mutation of the Stارد13 (Dк2) RhoCap gene due to retrovirial integration and that this gene plays a role in ceramide signaling to the RhoA pathway and control of the PGP synthase activity.

MATERIALS AND METHODS

MATERIALS

[U-14C]Glycerol-3-phosphate, [5-3H]cytidine 5'-triphosphate (CTP), and [32P]orthophosphate were obtained from GE Healthcare (Oakville, ON, Canada) or DuPont Canada (Mississauga, ON, Canada). Phosphatidyl[1-14C]Glycerol was synthesized from [1-14C]Glycerol-3-phosphate as described previously (Xu et al., 1999). Etoposide, tetrazolium salt (MTT), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), crystal violet, C2-Cer, and all lipid standards were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture media and reagents were products of Invitrogen (Burlington, ON, Canada). Thin-layer chromatographic plates (silica gel G) were from Fisher Scientific (Edmonton, AB, Canada). Antibodies to cleaved caspase-3 (catalog no. 9661) and poly (ADP-ribose) polymerase (PARP) (catalog no. 9548) were obtained from Cell Signaling Technology (Pickingen, ON, Canada).

Selection of the Etoposide-C2-Cer-resistant Cell Line E91

The CHO-K1 ceramide-resistant cell line E91 was isolated from a pool of etoposide resistant clones that had been previously infected with the U3NeoSV1 promoter trap retrovirus vector (Hicks et al., 1995). The CHO-K1 clone 22 cells also contain a transfected ecotopic retrovirus receptor gene (Hubbard et al., 1994). Cells were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 g/ml streptomycin, and 0.25 g/ml amphotericin. CHO-K1 cells were initially infected with helper virus-free U3NeoSV1 promoter trap retrovirus at a multiplicity of infection of 0.1. This virus vector contains the G418 resistance gene (Neo) in the U3 long terminal repeat (LTR) region of the virus (Hicks et al., 1995). Pools of G418-resistant colonies were then replated in the presence of the topoisomerase II inhibitor etoposide at a concentration of 5 mM dissolved in dimethyl sulfoxide (DMSO) for 2 d, and the media were then changed. Etoposide-resistant colonies were picked and replated in 10 mM etoposide in 24-well microtiter plates. After 1 wk of growth, the media was changed and the same population of uninfected parental CHO-K1 cells (5 x 10^6) and identical plating conditions, no etoposide-resistant colonies were obtained. Etoposide-resistant colonies were tested for parallel resistance to C2-Cer dissolved in absolute ethanol.

Cell Viability Assays

Short-term cell viability was determined by the MTT tetrazolium salt assay as described previously (Campling et al., 1991). For long-term survival, a clonal assay using crystal violet staining and dissolusion was as follows (Essmann et al., 2004). Briefly, 1000 cells were seeded on six-well plates, and cells were treated with drug or DMSO for 48 h. Media were removed, and cells were allowed to recover with media changes every 2 d for 1 wk. Adherent cells were stained with 0.5% aqueous crystal violet and washed, and then the stain was dissolved in 0.1% acetic acid in 50% ethanol. Triplicate samples were read on SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 558 nm. SOFTmax Pro3.1.2 software (Molecular Devices) was used to compile and analyze the data.

De Novo Phospholipid Biosynthesis Studies

Cells at confluence were made quiescent by incubation with a serum-free medium for 12 h before the addition of chemical. All cell incubation procedures were performed at 37°C. Cell viability was assessed by trypsin blue exclusion. Cells were incubated for 4 h with [32P]orthophosphate (20 Ci/mmol) in the absence or presence of 30 μM C2-Cer. Then, cells were harvested, and radioactivity incorporated into phospholipids was determined as described previously (Hatch, 1994; Hatch and McClarty, 1996).

Preparation of Mitochondrial Fractions and Assay of PGP Synthase Activity

Cells were cultured as described above, and all isolation procedures were performed at 4°C. Cells in 100-mm tissue culture dishes were incubated with 3 ml of medium in the absence or presence of 30 or 50 μM C2-Cer for 4 or 24 h. The medium was removed, and the cells were then washed twice with 5 ml of fresh medium. The cells were then harvested with 4 ml of ice-cold phosphate-buffered saline (PBS) by using a rubber policeman, and they were then placed into glass tubes. These cells were centrifuged at 1000 × g for 5 min. The PBS was removed, and a 10% homogenate prepared in 0.25 M Tris-HCl, pH 7.4, 0.145 M NaCl, and 1 mM EDTA by using 40 strokes of a tight-fitting Dounce A homogenizer. The homogenate was centrifuged at 1000 × g (RC-5 centrifuge with SS-34 rotor; Sorvall, New York, CT) for 10 min. The supernatant was centrifuged at 10,000 × g (RC-5 centrifuge) for 15 min. The pellet was resuspended in 1 ml of stabilization buffer (50 mM Tris-maleate, pH 6.5, 0.1% glycerol by volume, 0.1 M KC1, 10 mM MgCl2, and 0.5% Triton X-100 by volume) by using 15 strokes of a Dounce A homogenizer. The mitochondrial fraction was used to assay PGP synthase, phospholipase A2 (PLA2) and CDP-DG synthetase, CL synthase, and PA:CTP cytidylyltransferase activities as described previously (Hatch and McClarty, 1996; Xu et al., 1999). Marker enzyme analysis indicated that the mitochondrial fraction was contaminated with ~5% microsomal particles.

Rho Activation Assay

Rho activity in CHO cell lines was analyzed using the active Rho-GTP pull-down assay kit available from Cytoskeleton (Denver, CO) (Ren et al., 1999). CHO cells were grown in 60-mm dishes, and then they were serum starved for 12 h followed by addition of 25 mg/ml lysophosphatidic acid (LPA) for 30 min or 50 μM C2-Cer for 4 h. At the end of the treatment, cells were rinsed with ice-cold PBS, and 500 μl of lysate buffer was added to each dish. Cells were scraped, and cell lysates were prepared according to manufacturer’s protocol. After centrifugation at 10,000 × g for 10 min, supernatants were incubated with glutathione transferase (GST)-tagged rhokin Rho binding domain (RBD) peptide immobilized on agarose, and activated GTP-Rho bound to rhokin-agarose was detected by Western blot with anti-RhoA antibody and compared with total Rho in the extract.

Plasmid Rescue and 5’ Rapid Amplification of cDNA Ends (RACE) Polymerase Chain Reaction (PCR)

To identify the flanking sequences of the U3NeoSV1 provirus from E91 cells, genomic DNA was cut with Ec0RI or BstEII, ligated and transformed into
DHS" cells, and selected with kanamycin and ampicillin as described previously (Hicks et al., 1995). Plasmids were sequenced using primers NeoA, 5'-ATT TCG TGT TGT GCC GAC TCA-3' and NeoB, 5'-CGA ATA GCC TCT TCG ACC CAA-3'. To amplify the first Dcl2-Neo transcript, total RNA was extracted from E91 cells with RNAeasy mini kit (QIAGEN, Mississauga, ON, Canada) and resuspended in 30 µl of RNase-free water as described by the manufacturer. First-strand cDNA was synthesized with 2 µl of total RNA by using SuperScript RNaseH-reverse transcriptase (Invitrogen) followed by the Smart RACE cDNA amplification kit protocol (Clontech, Mountain View, CA). 5'-RACE PCR was performed using Smart universal primer mix and NeoGSP1 primer 5'-3'-GAA TAC TTT CTC GGC AGG AGC AAG GTG A-3' and neo 5'-CTT CCT TAC TTC GCC GCA-3', and sequenced using the ABI Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) by using Stard13 RhoGap primer or NeoA primers. Sequencing reactions were analyzed on an automated DNA sequencing machine (3730xL Genetic Analyzer; Applied Biosystems). The nucleotide sequence for Stand13 fusion cDNA has been deposited in the GenBank database under accession no. DQ191848.

Generation of Cell Lines Defective in RhoGAP and Rho Signaling

To knockdown the Dcl2 gene activity, we used two vector-mediated short hairpin RNA interference (shRNA) and synthetic small interfering RNAs (siRNA) to silence the Dcl2/Dlc2 and the RhoGap/RhoGap genes. To amplify the RNAeasy amplification kit protocol (Clontech, Mountain View, CA). 5'-RACE PCR was performed using Smart universal primer mix and NeoGSP1 primer 5'-3'-GAA TAC TTT CTC GGC AGG AGC AAG GTG A-3' and neo 5'-CTT CCT TAC TTC GCC GCA-3', and sequenced using the ABI Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) by using Stard13 RhoGap primer or NeoA primers. Sequencing reactions were analyzed on an automated DNA sequencing machine (3730xL Genetic Analyzer; Applied Biosystems). The nucleotide sequence for Stand13 fusion cDNA has been deposited in the GenBank database under accession no. DQ191848.

Quantitative Real-Time RT-PCR Analysis

Amplification of each target cDNA was performed with QuantiTect SYBR Green PCR Master Mix (QIAGEN) by using a Real-Time PCR Detection System iQ5 cycler (Bio-Rad, Hercules, CA) according to the protocols provided. The PCR cycling was programmed as 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles. PCR primers used were as follows: PCP sybhave forward, 5'-AAC AGC AGC TTC TGC TGC AGT AG-3'; reverse, 5'-GAA GTC TGC AAT CTC AGC ACA G-3'; and for GAPDH, forward, 5'-TGA AGG AAG GGT GGG AG-3' and reverse, 5'-TGA AGC AGG CAT CTT AGG G-3'. Relative gene expression was determined using the 2^−ΔΔCt method normalized to the GAPDH levels (Livak and Schmittgen, 2001). Real-time PCR data quality control, 2^−ΔΔCt, and analysis of covariance calculations were run on the SAS program as described (Yuan et al., 2006).

RESULTS

Etoposide-resistant E91 Cell Line Shows Cross-Resistance to C2-Ceramide

In a study to identify genes that may be involved in etoposide drug response, we used the U3NeoSV1 promoter trap retrovirus as mutagen and genetic tag. Virus-infected cells were selected for resistance to etoposide (see Materials and Methods). One etoposide-resistant clone was isolated, named E91, also showed cross-resistance to C2-ceramide compared with parental CHO-K1 CI22 and Neo610 cell lines (Figure 1, A and B). The Neo610 cells are U3NeoSV1-infected cells that were not selected for etoposide resistance. This line showed a slight resistance compared with the parental CHO-K1 CI22 cells but less than the E91 line. This indicates that expression of the Neo resistance gene did not have a major effect on etoposide response. In contrast, the E91 line showed reduced killing with etoposide up to a 300 µM concentration compared with CHO-K1 CI22 and Neo610 cells in MTT cell growth assays. CHO-K1, Neo610, and E91 cells were also tested for parallel resistance to C2-Cer. Cells were incubated for 48 h with various concentrations of C2-Cer, and the cell viability was determined by MTT assay (Figure 1B). Approximately 60–70% of CHO-K1 and Neo610 cells incubated with 10 µM C2-Cer for 48 h underwent cell death. In contrast, only 10% of E91 cells incubated with 10 µM C2-Cer for 48 h underwent cell death.

To determine whether this difference in cell survival was reflected in changes in apoptosis induction, we looked at cleavage of caspase-3 and PARP after various times and doses of etoposide (Figure 2, A and B). After 24 h of drug treatment, the E91 cells showed slightly higher levels of caspase-3 compared with the CI22 cell line. However, after 48 h of treatment, the E91 cells showed a slight reduction in the level of cleaved caspase-3 compared with the CI22 cells. Both cell lines showed similar levels of PARP cleavage at all time points. These results suggest that the survival difference between E91 and CI22 cells is not due to major changes in the level of apoptosis induction.
E91 Cells Are Defective in Ceramide Signaling

The above-mentioned studies suggested that ceramide signaling, as related to cell death, was impaired in E91 cells. To determine whether intracellular ceramide signaling was impaired in E91 cells, we examined a ceramide-regulated process not directly related to cell death. Previously, we had shown that addition of exogenous C2-Cer or TNF-α/H9251 to H9c2 cells resulted in elevated CL de novo biosynthesis via activation of PGP synthase and PLA2 activities (Xu et al., 1999). Therefore, we examined PGP synthase and PLA2 activities in CHO-K1 Cl22, Neo610, and E91 cells after incubation with exogenous C2-Cer. PGP synthase activities were elevated 57 and 46% in Cl22 CHO-K1 and Neo610 cells, respectively, after C2-Cer treatment (Table 1). In contrast, C2-Cer treatment of E91 cells did not elevate PGP synthase activity above the constitutive level. PLA2 activities were elevated 58% in CHO-K1 and 44% in Neo610 cells when treated with C2-Cer (Table 2). In contrast, C2-Cer treatment of E91 cells did not elevate PLA2 activity. Thus, ceramide signaling was impaired in the E91 cell line.

We next examined PGP synthase and PLA2 activities in CHO-K1, Neo610, and E91 cells incubated with exogenous TNF-α. PGP synthase activities were elevated 64% in CHO-K1 and 53% in Neo610 cells treated with TNF-α for 1 h, but they returned to baseline levels by 4 h (Figure 3A). TNF-α treatment of E91 cells did not affect PGP synthase activity at either time examined. PLA2 activities were elevated by 61% in CHO-K1 and 59% in Neo610 cells treated with TNF-α.
levels after C2-ceramide treatment. Cardiolipin synthetase activities were 2.9 ± 0.5 pmol/min/mg protein in CHO-K1 cells, and they were similar in the Neo610 and E91 cells. We previously demonstrated that addition of exogenous bacterial SMase to H9c2 cardiac cells, which would mobilize ceramide from the cell surface SM, did not affect PGP synthase or PLA2 activities (Xu et al., 1999). In agreement with our previous study, treatment of cells with exogenous bacterial SMase did not affect these enzyme activities in CHO-K1, Neo610, or CHO-E91 cells (data not shown). These data indicate that the elevation in PGP synthase and PLA2 activities in CHO-K1 and Neo610 cells were mediated by intracellular generated ceramide. The above-mentioned data suggest that ceramide insensitivity in E91 cells may be due to a defect in intracellular ceramide signaling.

**E91 Cells Show a Defect in RhoGap Activity**

We next determined where the promoter trap retrovirus was integrated in E91 cells and whether this contributed to the ceramide signaling defect. The Neo resistance gene located in the U3 LTR region on this virus can be activated by integration proximal to a promoter region or by splicing of the mRNA into the cryptic 3’ splice site in the Neo gene when integrated into an intron (Osipovich et al., 2004). Initially, we determined the flanking sequences of the U3NeoSV1 provirus through plasmid rescue as outlined in Materials and Methods. Sequencing of the flanking sequences revealed that the virus had integrated into intron 1–2 of the CHO Stard13 gene in E91 cells near the exon 1 (data not shown). This sequence is the orthologue of the mouse Stard13 RhoGap gene found on mouse chromosome 5 (GenBank accession no. BC027830). Next, we determined how the Neo gene in the provirus was activated using 5’ RACE. The sequences of this cDNA and predicted protein product are shown in Figure 4A. This sequence shows a fusion transcript of exon 1 of the Stard13 gene spliced into the cryptic 3’ splice site of the Neo gene. This fusion is similar to what has been described previously for activation of the Neo gene in the U3NeoSV1 retrovirus when integrated into introns of other genes (Osipovich et al., 2004).

To determine the expression of the other Stard13 allele in E91 cells, multiplex RT-PCR was carried out using primers that would recognize the normal and fusion transcripts along with GAPDH primers (Figure 4B). This gel shows RT-PCR products of 575 and 699 base pairs from CHO-K1 Cl22 RNA that are expected sizes of the normal Stard13 and GAPDH transcripts, respectively. In contrast, the E91 cells showed three products with a reduced level of the normal Stard13 PCR product and a 170-base pair fragment, which is the expected size of the fusion transcript of exon 1 with the Neo gene. These results indicate that the normal Start13 transcript is found at a reduced level in E91 cells compared with CHO-K1 Cl22 cells.

To determine whether E91 cells show reduced RhoGap activity, we measured the level of active GTP bound Rho protein by using the GST-RBD rhotekin pull-down assay (Ren et al., 1999) (Figure 5, A–C). These results show higher levels of the active GTP bound RhoA in the E91 cells compared with the parental CHO-K1 Cl22 cells after both LPA and C2-ceramide treatments (Figure 5, A–C). This is consistent with there being reduced RhoGap activity in E91 cells. The parental CHO-K1 Cl22 cells showed only a slight increase or a reduction in active RhoA after treatment, consistent with higher RhoGap activity.

**RhoGAP Mediates Ceramide Activation of PGP Synthase**

To directly test whether reduced expression of Stard13 is involved in induction of PGP synthase, we transected...
CHO-K1 Cl22 cells with plasmids expressing shRNAi to knock down Stard13 expression. The level of RNA knockdown was determined by RT-PCR and compared with GAPDH as an internal control (Figure 6). These results show good RNA knockdown for cell lines RSC5 and -6 (Figure 6A, lanes 4–6). The RSC1 line showed a faint Stard13 band. Under these PCR conditions, E91 cells showed very low expression of the normal Stard13 allele. There was also evidence of some off-target effects on expression because the control cell lines expressing both the mutated RhoGap (RhoGapMT; Figure 6A, lane 7) or shRNAi to GFP (both specific and mutant shRNAi; Figure 6B, lanes 3 and 4) showed a slight reduction in both Stard13 and GAPDH levels compared with Cl22 or Cl22 cells expressing GFP alone. These lines were used for further study.

The active Rho levels in the Stard13, shRNAi knockdown cell lines were measured by the Rho-GTP pull-down assay (Figure 5, A–C). The RSC5 and -6 lines expressing Stard13-specific shRNAi showed significant increases of GTP bound Rho-GTP (Figure 5A and B). Blots were probed with monoclonal antibody for RhoA. Representative immunoblots are shown with RBD-GST bound Rho (top) and total Rho from a portion of the same extract (bottom). (C) The graph shows the average ratio of GTP-bound Rho to the total amount of Rho as determined in the pull-down assays. Results are the average three experiments ± SD (*p < 0.05 vs. untreated control).

Figure 4. Fusion of exon 1 of Stard13 into the cryptic Neo 3' Splice Site in the E91 cell line. (A) cDNA sequence of the fusion transcript. The cDNA sequence was generated by 5' RACE and sequenced using NeoA- and Stard13-specific primers. Amino acid single-letter codes are shown for the Stard13 exon 1 sequence fused to the Neo coding sequence. The arrows indicate the location of the spliced transcript that uses the Neo 3' SS and the Stard13 exon 1 and Neo boundaries. (B) Multiplex RT-PCR of RNA from CHO-K1 Cl22 and E91 cell lines. RT-PCR was performed using primers for Stard13 exon 1–5, Neo gene, and GAPDH in the same reaction. MW, molecular weight marker.

Figure 5. Increased Rho-GTP in Stard13 shRNAi-expressing cells. Cell lines were serum starved for 12 h, and C2-ceramide was added for 4 h (A) or LPA was added for 30 min (B). Rho-GTP levels were measured using pull-down assays (see Materials and Methods for details). Blots were probed with monoclonal antibody for RhoA. Representative immunoblots are shown with RBD-GST bound Rho (top) and total Rho from a portion of the same extract (bottom). (C) The graph shows the average ratio of GTP-bound Rho to the total amount of Rho as determined in the pull-down assays. Results are the average three experiments ± SD (*p < 0.05 vs. untreated control).

Figure 6. Multiplex RT-PCR of cell lines expressing shRNAi plasmids. (A) Multiplex RT-PCR was carried out using primers for Stard13 and GAPDH cDNAs as outlined in Materials and Methods. Lane 1, molecular weight marker; lane 2, CHO-K1 Cl22 cell line; lane 3, E91 cell line; lane 4, RSC1 cell line; lane 5, RSC5 cell line; lane 6, RSC6 cell line; and lane 7, RhoGapMT cell line. Cell lines RSC1 and -6 contain the pSuper plasmid expressing Stard13 shRNAi. Cell line RSC5 contains the pSuperior.puro plasmid expressing Stard13 shRNAi, which is tetracycline inducible and pcDNA6/TR containing the Tet repressor. This line was grown in the presence of tetracycline. Cell line RSC6 contains the pSuper.neo + GFP plasmid expressing Stard13 shRNAi. RhoGapMT contains pSuper expressing a mutated Stard13 shRNAi. (B) RT-PCR of control cell lines: lane 1, marker; lane 2, CHO-GFP contains pEGFP plasmid; lane 3, CHO-GFP RNAi contains pEGFP + pSuper expressing GFP shRNAi; and lane 4, CHO-GFPMT contains pGFP + pSuper plasmid expressing a mutated GFP shRNAi.
experiments/ 

treated in 

for 24 h, and PGP synthase activity was then determined as described in Materials and Methods. The plotted 2^−ΔΔCt results are based on the mean of three replicates at three RNA concentrations ± SD (p < 0.0001 and **p < 0.001, as determined by ANOVA compared with the untreated control). (C) Correlation of PGP synthase RNA induction versus the active GTP-bound Rho after ceramide induction. The active Rho A compared with nontreated cells, which was comparable with the E91 cells after treatment with LPA or C2-ceramide. The RSC1 line showed increased active Rho compared with nontreated cells, but this did not reach significance. The control cell line expressing a mutated Stard13 shRNAi (RhoGapMT) showed reduced levels of active Rho activity comparable with the parental CHO-K1 CI22 cells after addition of LPA or C2-ceramide.

These shRNAi-expressing cell lines were then tested for induction of the PGP synthase enzyme activity after C2-ceramide treatment (Figure 7A). CHO-K1 CI22 and shRNAi control line RhoGapMT showed significant increases in PGP synthase activity after C2-Cer addition (33.2 and 32%, respectively). The RSC1 and RSC5 shRNAi-expressing lines showed smaller nonsignificant increases of PGP synthase activity after C2-Cer treatment (10 and 13%, respectively) compared with CHO-K1 CI22 cells. In contrast, the E91 and RSC6 lines showed a reduction of PGP synthase activity after ceramide treatment compared with untreated cells (−16 and −26%, respectively), with the RSC6 cells showing a significant reduction.

These cells were then tested for changes in PGP synthase mRNA expression after C2-Cer treatment by using real-time RT-PCR (Figure 7B). The CHO-K1 CI22 and RhoGapMT lines showed significant 2.5- and 3.2-fold induction of PGP synthase mRNA, respectively, after C2-Cer treatment. E91 cells exhibited only a 1.2-fold induction of PGP synthase mRNA expression after ceramide treatment. The Stard13 knockdown cell lines RSC1 and RSC5 showed significant induction at 2.0- and 1.7-fold; however, this was less than that seen in the CI22 and RhoGapMT cells. The induction of PGP synthase mRNA in the line RSC6 cells showed a nonsignificant 1.3-fold induction of PGP synthase mRNA after C2-ceramide addition, similar to the E91 line. Because there was variation in knockdown of Stard13 as reflected in the levels of active GTP-RhoA (Figure 5C), we wanted to determine whether PGP synthase RNA induction was correlated with the level of active GTP-bound Rho in cells after C2-ceramide treatment. This analysis (Figure 7C) shows that there is a linear relationship between active GTP-bound Rho and PGP synthase mRNA induction after ceramide treatment. Thus, the higher the GTP-Rho levels in the cell the lower the PGP synthase mRNA levels, i.e., a smaller ΔΔCt value. We have also confirmed these results by using siRNAs targeted to Stard13 mRNA with a mutated Stard13 control transfected into CI22 cells (Supplemental Figure 1).

The previous results suggest that reduced Stard13 RhoGap activity in cells results in increased active RhoA, which in turn interferes with ceramide induction of PGP synthase. To further test the role of Rho, CI22 and E91 cell lines stably expressing a dominant-negative RhoA (S19N) or vector control (pZIPneo) were isolated, and PGP synthase activity after C2-ceramide induction was determined (Figure 8). In these experiments, 3% fetal calf serum was included in the medium because serum starvation of the RhoA(S19N)-expressing cells was lethal. The results in Figure 8A show that CI22 cells with stable expression of dominant-negative RhoA increased the induction level of PGP synthase activity after C2-ceramide compared with the empty vector control cells (135 vs. 76%). E91 cells expressing RhoA19N also showed a significant 29% increase in PGP synthase activity ratio data were taken from Figure 5 and plotted against the ΔΔCt values. The line and 95% confidence bands was determined by linear regression analysis (R = 0.93795 and p = 0.00566).
after ceramide addition in contrast to the vector control cells that showed a slight reduction (−5%).

The constitutive level of PGP synthase in the RhoA(S19N)-expressing E91 cells was significantly reduced compared with the vector only controls cells. Therefore, we wanted to confirm these results in E91 cells transiently expressing the dominant-negative RhoA(S19N) plasmid (Figure 8B). These results show that the constitutive levels of PGP synthase were slightly reduced compared with the vector control cells, but this did not reach significance. C2-treated cells transfected with 750 ng of RhoA(S19N) showed a significant 24% increase in PGP synthase activity after 24 h compared with the transfected but nonceramide-treated cells. This is in contrast to the vector-only transfected cells that showed a nonsignificant reduction (−3.2%) in PGP synthase activity after ceramide treatment compared with untreated cells. These results demonstrate that interference with Rho signaling in E91 cells could reverse the inhibition of PGP synthase induction after ceramide treatment.

**DISCUSSION**

This study is the first report for the regulation of a phospholipid biosynthetic enzyme through RhoGap expression. The results of this study support the concept that ceramide may alter RhoA activity via interaction with the Stard13 RhoGap. Elevated GTP-bound RhoA levels found in cells with a mutated Stard13 gene or knock down of its mRNA can dampen ceramide’s induction of PGP synthase gene expression. Also, cells with elevated active RhoA show partial resistance to the drug etoposide.

Stard13 is unique as a RhoGap in that it also contains a steroidogenic acute regulatory protein (StAR)-related lipid transfer domain (START) and a SAM2-interacting domain (Soccio and Breslow, 2003). The prototypical START-containing protein is the STAR, which functions to transport cholesterol from the outer to inner mitochondria membranes.
in steroidogenic cells (Stocco, 2001). Whether the START domain of Stard13 directly interacts with ceramide awaits future experiments. However, there is a precedent for ceramide interacting with a Start lipid domain. The CERT (Stard 11) protein, which contains both a START lipid transfer and a phosphatidylinositol-4-monophosphate binding domains, functions to shuttle ceramide from the endoplasmic reticulum to the Golgi (Hanada et al., 2003). Also of relevance is the recent finding that the START domain of Dlc-2 plays a role in its localization to mitochondria and proximity to lipid droplets found in the cytoplasm (Ng et al., 2006).

Three RhoGap proteins with START domains have been described in mammals. These are Dlc-1 (also named p122RhoGap, Argap57), Stard13 (Dlc-2), and Stard8 (KIAA0189 RhoGAP) (Soccio and Breslow, 2003). The Stard13 (Dlc-2) gene is frequently found deleted in hepatocellular and breast carcinoma cells (Ching et al., 2003; Nagraja and Kandpal, 2004), and along with the closely related Dlc-1 is a tumor suppressor gene (Yuan et al., 1998; Ng et al., 2000).

The experiments presented here suggest that the START lipid binding domain of the Stard13 may be interacting with endogenous ceramide to stimulate its RhoGap activity and then down-regulate RhoA activity. This in turn would alter RhoA’s downstream effec tors and result in a change in CL metabolism through alteration of the PGP synthase and PLA2 activities. E91 and Stard13 RNAi knockdown cells showed reduced ceramide activation of these enzymes, suggesting that active RhoA inhibits this process partially through control of transcription of the PGP synthase gene. Because the magnitude of PGP synthase activity did not fully reflect the mRNA induction levels, this would suggest that there is a posttranslational control of PGP synthase as well. Posttranslational control of PGP synthase by inositol has been described in yeast, and this negative regulation is mediated through changes in phosphorylation of the protein (He and Greenberg, 2004). Our results predict that increased ceramide levels would result in lower RhoA activity in normal cells through stimulation of RhoGap activity, and this may be mediated through ceramide binding to the START lipid domain. With these mutant cells, we will be testing this hypothesis and try to understand which downstream Rho effectors are involved in this control.

The E91 cells with a retrovirus integrated into one allele of the Stard13 gene showed reduced expression of the other allele and increased levels active GTP-bound RhoA after ceramide and LPA treatment. This may be due to the fact that CHO cells are functionally hemizygous for many loci due to either increased promoter methylation or chromosomal rearrangements (for reviews, see Siminovitch, 1976; Holliday, 1991). This was the reason why we chose CHO cells for the promoter trap experiments because only one allele would need to be inactivated for many genes.

Our results showed an association with etoposide resistance in cells with knockdown or mutation of Dlc-2. The RSC6 line showed resistance at levels comparable with the E91 cells, and it also showed the high levels of active RhoA, whereas the RSC1 line that showed only slight resistance and lower levels of active RhoA. This may indicate that there is a critical threshold of active RhoA in cells that is needed to manifest etoposide resistance.

The results testing ceramide sensitivity were ambiguous. Although the RNAi knockdown of Stard13 mRNA resulted in increased ceramide resistance compared with shRNAi controls, this did not reach significance except for one cell line. The shRNAi control cells showed increased sensitivity to ceramide compared with the parental C122 cells. This may be due to possible off target effects of RNAi such as the production of interferon (Bridge et al., 2003). These off target effects of RNAi did not seem to affect the ceramide signaling to PGP synthase to the same extent. These results also emphasize the importance of appropriate controls for RNAi experiments especially with respect to drug response. We will have to wait the generation of knockout mice to better test the role of Stard13 in ceramide sensitivity.

Others have found association of increased Rho activity and drug resistance. One group found that dominant-negative RhoA expression could interfere with resistance to etoposide induced apoptosis caused by CD44 overexpression (Fujita et al., 2002). Another study found that HMG-CoA reductase inhibitors simvastatin and lovastatin could reverse the cell adhesion-mediated drug resistance to drugs such as malphalan, treosulfan, and doxorubicin in human multiple myeloma cells (Schmidmaier et al., 2004). This reversal of resistance by statins seemed to be due to interference with Rho protein geranylgeranylation (Schmidmaier et al., 2004). Elevated expression of the Lymphoid blast crisis protooncogene, a Rho guanine-nucleotide exchange factor, in hepatocarcinoma cells results in resistance to doxorubicin (Sterpetti et al., 2006). Our results suggest that tumors with mutation of the Stard13 (Dlc-2) gene may be intrinsically resistant to some drugs.

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