

Research article

## REGULATOR OF G-PROTEIN SIGNALLING EXPRESSION AND FUNCTION IN OVARIAN CANCER CELL LINES

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**Abstract:** Regulator of G-protein signalling (RGS) proteins critically regulate signalling cascades initiated by G-protein coupled receptors (GPCRs) by accelerating the deactivation of heterotrimeric G-proteins. Lysophosphatidic acid (LPA) is the predominant growth factor that drives the progression of ovarian cancer by activating specific GPCRs and G-proteins expressed in ovarian cancer cells. We have recently reported that RGS proteins endogenously expressed in SKOV-3 ovarian cancer cells dramatically attenuate LPA stimulated cell signalling. The goal of this study was twofold: first, to identify candidate RGS proteins expressed in SKOV-3 cells that may account for the reported negative regulation of G-protein signalling, and second, to determine if these RGS protein transcripts are differentially expressed among commonly utilized ovarian cancer cell lines and non-cancerous ovarian cell lines. Reverse transcriptase-PCR was performed to determine transcript expression of 22 major RGS subtypes in RNA isolated from SKOV-3, OVCAR-3 and Caov-3 ovarian cancer cell lines and non-cancerous immortalized ovarian surface epithelial (IOSE) cells. Fifteen RGS transcripts were detected in SKOV-3 cell lines. To compare the relative expression levels in these cell lines, quantitative real time RT-PCR was performed on select transcripts. RGS19/GAIP was expressed at

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Abbreviations used:  $C_T$  – threshold cycle; ELISA – enzyme-linked immunosorbent assay; GPCR – G-protein coupled receptor; GAPDH – glyceraldehyde-3-phosphate dehydrogenase;  $GRO\alpha$  – growth-regulated oncogene  $\alpha$ ; IOSE – immortalized ovarian surface epithelial; IP – inositol phosphates; LPA – lysophosphatidic acid; PLC – phospholipase C; RGS – regulator of G-protein signalling; RT-PCR – reverse transcriptase polymerase chain reaction; siRNA – small interfering RNA

similar levels in all four cell lines, while RGS2 transcript was detected at levels slightly lower in ovarian cancer cells as compared to IOSE cells. RGS4 and RGS6 transcripts were expressed at dramatically different levels in ovarian cancer cell lines as compared to IOSE cells. RGS4 transcript was detected in IOSE at levels several thousand fold higher than its expression level in ovarian cancer cells lines, while RGS6 transcript was expressed fivefold higher in SKOV-3 cells as compared to IOSE cells, and over a thousand fold higher in OVCAR-3 and Caov-3 cells as compared to IOSE cells. Functional studies of RGS 2, 6, and 19/GAIP were performed by measuring their effects on LPA stimulated production of inositol phosphates. In COS-7 cells expressing individual exogenous LPA receptors, RGS2 and RSG19/GAIP attenuated signalling initiated by LPA1, LPA2, or LPA3, while RGS6 only inhibited signalling initiated by LPA2 receptors. In SKOV-3 ovarian cancer cells, RGS2 but not RGS6 or RGS19/GAIP, inhibited LPA stimulated inositol phosphate production. In contrast, in CAOV-3 cells RGS19/GAIP strongly attenuated LPA signalling. Thus, multiple RGS proteins are expressed at significantly different levels in cells derived from cancerous and normal ovarian cells and at least two candidate RGS transcripts have been identified to account for the reported regulation of LPA signalling pathways in ovarian cancer cells.

**Key words:** Regulator of G-protein signalling (RGS) proteins, Ovarian cancer, SKOV-3, OVCAR-3, Caov-3, IOSE, RT-PCR

## INTRODUCTION

Lysophosphatidic acid (LPA) is a key mediator of ovarian cancer initiation and progression, functioning as an autocrine activator of proliferation [1, 2], survival from apoptotic signals [3], migration [4], production of angiogenic growth factors [5], and production of proteases critical for metastasis [6]. LPA is released from ovarian cells and accumulates in ascitic fluid where it is then available to bind and activate a family of cell surface G-protein coupled receptors (GPCRs). Three related, well-characterized "classic" LPA receptors, LPA1, LPA2, and LPA3 (previously named Edg-2, Edg-4, and Edg-7, respectively) are expressed on the surfaces of ovarian cancer cells and mediate responses to LPA [7]. Two additional receptors, LPA4 and LPA5, which are related to each other but not to the "classic" LPA receptors have also recently been described [8, 9]. Each of these receptors functions by activating heterotrimeric guanine nucleotide binding proteins (G-proteins) in an LPA dependent manner. Thus, the central mediators of signalling by LPA receptors are G-proteins. LPA binding to its receptors stimulates the G-protein to undergo nucleotide exchange, switching from the inactive di-phosphate (GDP) bound form to the active tri-phosphate (GTP) bound form. It is the active GTP bound G-protein that initiates cell signalling cascades to ultimately regulate cancer cell function. G-proteins slowly return to the inactive state by auto-hydrolysis of GTP [10].

Regulator of G-protein signalling (RGS) proteins function to deactivate heterotrimeric G-proteins by dramatically accelerating the rate at which  $G\alpha$  subunits hydrolyze GTP [11-13]. RGS proteins have been shown to have profound effects on the kinetics and magnitude of *in vivo* receptor signalling pathways [11, 12]. Over the past decade, RGS proteins that deactivate G-proteins have been demonstrated to acutely regulate signalling downstream of GPCRs. We recently demonstrated that RGS proteins dramatically regulate LPA signalling in ovarian cancer cells using a mutagenesis strategy to compare signalling activity of  $G\alpha_i$  proteins that were either wild-type with respect to RGS regulation or insensitive to RGS regulation. LPA signalling in SKOV-3 ovarian cancer cells was significantly more robust in cells expressing RGS insensitive  $G_{i2}$ -proteins than in cells expressing wild-type RGS sensitive G-proteins with respect to inhibition of adenylyl cyclase and cellular migration. This suggests that endogenous RGS proteins expressed in SKOV-3 ovarian cancer cells normally attenuate signalling of  $G\alpha_i$ -mediated LPA-stimulated signalling [14].

The goal of the current study was to identify candidate RGS proteins expressed in SKOV-3 cells that may regulate G-protein signalling and second, to determine if these RGS protein transcripts are differentially expressed among commonly utilized ovarian cancer cell lines and non-cancerous ovarian cell lines. We identified multiple RGS protein transcripts in SKOV-3 cells that may account for negative regulation of LPA signalling pathways and determined that two of these RGS transcripts are expressed at dramatically different levels in cell lines derived from normal versus cancerous ovarian cells. We further determined the ability of these RGS proteins to attenuate signalling from individually expressed exogenous LPA receptors and LPA receptors endogenously expressed in SKOV-3 and CAOV-3 ovarian cancer cells.

## METHODS AND MATERIALS

### Cell culture

Human OVCAR-3, SKOV-3, Caov-3, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and grown according to ATCC recommendations. Immortalized ovarian surface epithelial cells (IOSE) were a kind gift from Dr. David Puett (University of Georgia).

### RNA isolation

RNA was isolated from tissue samples and cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Monolayers IOSE, Caov-3, SKOV-3, and OVCAR-3, and HeLa cervical cancer cells were homogenized in 1 ml of Trizol reagent per 10 cm<sup>2</sup> of plate surface area by passing the lysates through a pipette tip several times. RNA preparations were treated with DNase, and the RNA was separated from the DNase using an RNEasy column (Qiagen). RNA preparations were aliquoted and stored at -80°C.

### **RT-PCR**

The RNA was analyzed for specific transcripts with Superscript One-step RT-PCR kit (Invitrogen) according to the manufacturers instructions. Primers were designed and analyzed for specificity, secondary structure, and dimerization using Vector NTI Software (Invitrogen). PCR conditions such as annealing temperature were optimized for each primer pair, and plasmid DNA (50 ng) encoding each target RGS sequence was used as a positive control. Reactions included forty cycles of amplification. Products of amplification were resolved by electrophoresis in 1.5 % agarose gels and visualized with ethidium bromide staining.

### **qRT-PCR**

To compare the level of expression of RGS transcript between cell lines, DNA was first synthesized from 1 $\mu$ g of total RNA using the High Capacity Reverse Transcriptase cDNA kit (Applied Biosystems) to amplify mRNA. Reactions without reverse transcriptase were also run as a control for DNA content. qPCR reactions were prepared using Applied Biosystem's Taq-man Gene Expression Assays and Universal PCR Mastermix according to the manufacturer's instructions. Reactions for GAPDH were performed in each RNA set as an internal standard. The PCR reaction was carried out in a Biorad iCycler using program parameters provided by Applied Biosystems. Briefly, 50 ng cDNA was provided as template with 2X Universal PCR Mastermix for 50 cycles of 15 second melt at 95°C and 1 minute of anneal/extension at 60°C. The primers in Taq-man Gene Expression Assays are designed to use the same annealing temperature. Threshold cycle ( $C_T$ ) values were determined for each transcript using the automated threshold function of the iCycler software, and data are reported as  $2^{-\Delta\Delta C_T}$  [15], using GAPDH housekeeping gene as internal controls and reporting data as expression in the ovarian cancer cells relative to IOSE cells.

### **DNA constructs and transfections**

Plasmids encoding RGS and receptor proteins were obtained from the UMR cDNA Resource Center (Rolla, MO). Transfections were performed using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions, at a ratio of 2  $\mu$ l of Fugene 6 reagent to 1  $\mu$ g plasmid DNA. Inositol phosphate experiments were carried out in 24-well dishes, and were transfected with 500 ng/well total plasmid DNA. COS-7 cells were transfected with 100 ng/well of receptor-encoding DNA, and the amount of RGS DNA per well was determined empirically to yield equivalent expression levels (see results), with the total amount of DNA per well being brought up to 500 ng with vector. ELISA experiments were performed in 96-well dishes, and cells were transfected with vector, receptor, and RGS plasmid DNA in proportional amounts relative to the 24-well dishes up to 100 ng total plasmid DNA/well.

**Inositol phosphate assay**

Production of inositol phosphates (IP) was quantified using established protocols [16]. Briefly: To measure IP production by PLC activation, COS-7 cells were plated in 24-well dishes at 50,000 cells/well. 48 hours prior to the assay, cells were transfected with the appropriate genes. Cells were labeled with 0.5  $\mu\text{Ci}/\text{well}$   $^3\text{H}$  myo-inositol (American Radiolabeled Chemicals, St. Louis, MO) for 18 hours to label the cellular pool of phosphatidyl inositol. The cells were treated with Oleoyl (18:1) LPA (Avanti Polar Lipids, Alabaster, AL) in the presence of 10 mM lithium chloride to inhibit degradation of inositol phosphates for 30 minutes at 37°C. Cells were then lysed in cold formic acid and neutralized with ammonium hydroxide, and the lysates were then loaded onto columns of AG 1-X8 anion exchange resin (Biorad, Hercules, California). The columns were washed with water and dilute ammonium formate to remove unhydrolyzed lipids. The  $^3\text{H}$  IPs were then eluted with 1.2 M ammonium formate/0.1 M formic acid, and added to scintillation fluid for counting. In some experiments, cells were treated with 200 ng/ml pertussis toxin (Sigma-Aldrich, St. Louis, MO) for 12 hours prior to IP assay.

**Western blotting**

Protein expression was determined using standard techniques. Cells were harvested and lysed in protein sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted using HA-epitope polyclonal primary antibodies and peroxidase-conjugated secondary antibodies (Bethyl laboratories, Montgomery, Texas), and visualized using SuperSignal Chemiluminescent substrate (Pierce, Rockford, Illinois).

**Cell-surface ELISA**

LPA receptor expression constructs used in these experiments contained an N-terminal (extra-cellular) Hemagglutinin (HA) tag that was used to detect receptors expressed at the cell surface. Cells were plated and transfected simultaneously with cells to be assayed in the inositol phosphate assays in 96-well plates. 48 hrs after transfection, on the day the second messenger assays were performed, cells were fixed with freshly prepared 4% paraformaldehyde for 10 minutes at 4°C. Cells were then washed once with Tris-buffered saline (TBS) and incubated sequentially in: blocking reagent for 1 hour (3% BSA in TBS); primary antibody for 2 hours (Rabbit anti-HA diluted 1:1000 in 1%BSA/TBS); TBS for 5 minutes 3 times; secondary antibody for 1 hour (Goat anti-Rabbit IgG HRP in 1% BSA/TBS); TBS for 5 minutes 3 times; and liquid substrate for 15 minutes [Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Sigma]. The optical density at 405 nm was determined in a plate reader (Victor, Perkin Elmer).

## RESULTS

### RGS expression in ovarian cell lines

In order to identify candidate RGS proteins that may be responsible for the observed negative regulation of signalling in SKOV-3 cells, we evaluated the expression of transcripts of RGS proteins in SKOV-3 ovarian cancer cells. Further, we compared RGS transcript expression in SKOV-3 cells to two other commonly studied ovarian cancer cell lines, OVCAR-3 and Caov-3, as well as immortalized ovarian surface epithelium (IOSE) cells derived from non-cancerous cells. Reverse transcriptase-PCR was performed on total RNA isolated from SKOV-3, Caov-3, OVCAR-3, and IOSE cell lines. High quality RNA was isolated and screened for RGS transcript expression as described in Methods using a panel of primers for 22 distinct RGS transcripts belonging to the five major subfamilies of RGS proteins.

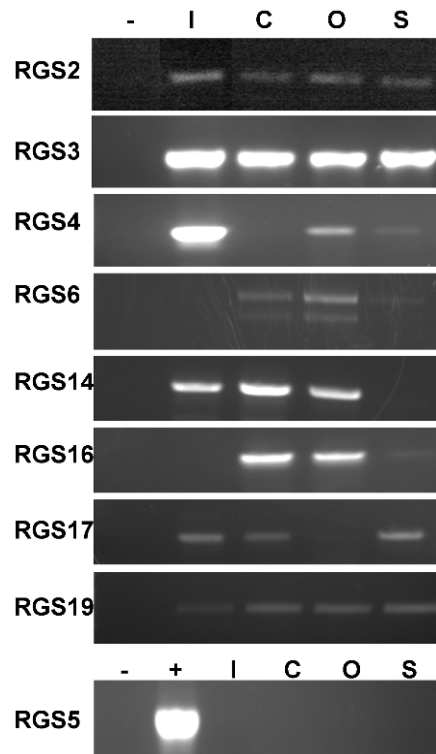


Fig. 1. RGS protein transcript expression in ovarian cell lines. RNA was isolated from IOSE cells (I), Caov-3 cells (C), OVCAR-3 cells (O), or SKOV-3 cells (S) using Trizol reagent (Invitrogen) and amplified using Superscript One Step RT-PCR kit (Invitrogen) according to manufacturer's instructions. Positive control reactions (+) contained plasmid DNA encoding the target RGS as template. Negative control reactions (-) contained no RNA or DNA template. Eight representative data images are shown. Results are representative of three separate experiments. Full results are summarized in Tab. 1.

Tab.1. Summary of RT-PCR data. I: IOSE, S: SKOV-3, O: OVCAR-3, C: Caov-3. +: Transcript consistently detected in RNA. -: Transcript not detected in RNA.

RGS protein	RT-PCR				G-protein target	Primers
	I	S	O	C		
Family A, RZ						
RGS17	+	+	-	+	i/o	3':TCAGAAGAAGAGCCAGCAGTAC 5':ATGGAGAGTATCCAGGTCCTAGAG
RGS19/GAIP	+	+	+	+	i/o	3':TAGGCCTCGGAGGAGGACTGTGATG 5':CCGCATGAGGCTGAGAAGCAGATC
RGS20/RGSZ	+	+	+	+	i/o/z	3':CAGCAGAGTTCATGAATCGAGG 5':ATGGGATCAGAGCGGATGGAGATG
Family B, R4						
RGS1	-	-	-	-	i/o, q/11	3':TATCTGATTTGAGGAACCTGGG 5':ATGCCAGGAATGTTCTTCTCTG
RGS2	+	+	+	+	q/11>i/o	3':GTCCTGGTAGAATTCTGACTCCAAG 5':GCAAAGTGCTATGTTCTTGGCTGTTC
RGS3	+	+	+	+	i/o, q/11	3':TGAGGGACCAGGGAAGCACAGTCTG 5':TTGCAGGCTGCGCGCTTCTTG
RGS4	+	+	+	-	i/o, q/11	3':TAAAACTCAGAGCGCACAAAGCGAG 5':AAGGACTTGCAGCTTTGCCCACTC
RGS5	-	-	-	-	i/o, q/11	3':TCCTCTGGCTTTGGGACAGCAGATC 5':CCTTACTGATGCCACGCAGGAACAAAG
RGS8	-	-	-	-	i/o, q/11	3':CAGAAACTGTTGTTGGACTGCATAG 5':GATGTGCAGAGATGAATCTAAGAGGC
RGS13	-	-	-	-	i/o, q/11	3':TGCAGCTGGACAGAGTGGCAGAGGC 5':ACCACCTGCCTGGAGAGAGCCAAAG
RGS16	-	-	+	+	i/o, q/11	3':ATAACCAAATGGCAACGTCTGATTG 5':GCAGAAACCTGAGTTTCATGAAGACACC
RGS18	-	-	-	-	i/o, q/11	3':AAGCGGGGACTCATCTTCTTCTGG 5':GCCAAGGACATGAAGAACAAGCTGGG
Family C, R7						
RGS6	-	+	+	+	i/o	3':TCTTCTTGGCCAGCAGCAAATCCTG 5':GCTCAAGGATCCGGGGATCAAAGAG
RGS7	+	+	+	+	i/o	3':TTAGTGCTGGCCCTCAGTGTGGTG 5':CCAGGGGAATAATTATGGGCAGACC
RGS9	+	+	+	+	i/o	3':TGGCATCGTTGGATGAGAAGTGTCTC 5':GCCGAGGATGGCATTCTCCAAAAG
RGS11	+	+	+	+	i/o	3':CTTTCATCTCCAGCGGGATCCAG 5':TGCCATCTACCTGGCCAAGAAGAAC
Family D, R12						
RGS10	+	+	+	+	i/o, q/11	3':TTAGCTGCAGTTTGAGCATCAGGC 5':ATGGAACACATCCACGACAGCGATG
RGS12	+	+	+	+	i/o	3':GCCATCGGTGGAATCTGTGTAAGAG 5':AAACAAAGATCCCTTTCGGAGTCGG
RGS14	+	-	+	+	i/o	3':GTCACAGGTCTGCCGCTTTCAGTG 5':GAGAGTCTCAGGGCTCCCTCAACTC
Family F, GEF						
p115-RhoGEF	+	+	+	+	12/13	3':CTTCTTGGTCTTGGCAGGCTCGTCC 5':ATCGGGGCTGAGGATGAGGATTTTG
PDZ-RhoGEF	+	+	+	+	12/13	3':AGCAGCATCCATGTCAACATCACTG 5':TGGGCAGCCTGTATGGTGAAAATGAC
LARG	+	+	+	+	12/13	3':GTCAGTCCCATACTACGTTTCTGCC 5':GTTACAGAAAGAACAGGAACGGCTAC

Overall, fifteen RGS transcripts were detected in SKOV-3 cells, and twelve of these (RGS2, 3, 4, 6, 7, 9, 10, 11, 12, 19, and 20) are known to deactivate -proteins of the Gi/o family [17]. Distinct patterns of expression were observed across the four ovarian cell lines tested. RGS transcripts segregated into three categories with respect to their expression in ovarian cancer cells. The first, largest category includes twelve RGS transcripts consistently detected in all four cell lines evaluated: RGS2, 3, 7, 9, 10, 11, 12, p115RhoGEF, PDZ-RhoGEF, and LARG. The second category includes five RGS transcripts that could not be detected in any of the ovarian cell lines evaluated, although the primers effectively amplified product in positive controls: RGS 1, 5, 8, 13, and 18. Finally, the most interesting category includes RGS transcripts that were consistently detected in some but not all of the ovarian cell lines. RGS4 was not detected in Caov-3 RNA, but was detected in the other three cell lines, while RGS6 was detected in all three ovarian cancer cell lines, but no amplification product was observed in IOSE RNA. (The double bands observed with RGS6 amplification are expected based on reported splice variants [18]). RGS14 transcript was consistently detected in IOSE, Caov-3, and OVCAR-3 cells, but not in SKOV-3. Similarly, RGS16 amplification product was not consistently detected in IOSE and SKOV-3 RNA, but was detected in OVCAR-3 and CAOv-03 RNA, and RGS17 transcript was not consistently detected in OVCAR-3 RNA, but was detected in the other three cell lines. A full summary of results is shown in Tab. 1 and representative data images (RGS2, 3, 4, 5, 6, 14, 16, and 19) are shown in Fig. 1.

### **Quantitative comparison of RGS expression**

Several RGS primer pairs consistently produced amplification products of different intensity from the different cell lines (e.g. RGS4); however, the conditions used in our RT-PCR analysis were highly sensitive to detect low-copy number transcripts (40 cycles of PCR amplification), which limited quantitative comparison between amplification products. Thus, we further evaluated the relative expression of select RGS transcripts between cell lines using real time quantitative RT-PCR. Taqman Gene Expression Assays (Applied Biosystems) were used to compare expression of RGS2, 4, 6, 14, and 19 transcripts to the internal standard GAPDH and to further determine their relative expression in cancer cell lines as compared to IOSE cells [15]. Threshold cycle ( $C_T$ , the cycle number in which fluorescence signal crosses threshold value) was determined for each transcript in each RNA preparation. The difference between RGS and GAPDH  $C_T$  values ( $\Delta C_T$ ) was determined for each cell line, and the difference between  $\Delta C_T$  values for IOSE and each cancer cell line was then determined ( $\Delta\Delta C_T$ ) (Tab. 2). This value was used to calculate positive fold changes in gene expression ( $2^{-\Delta\Delta C_T}$ ) and negative fold changes ( $-1/2^{-\Delta\Delta C_T}$ ).



Tab. 2. Summary of quantitative RT-PCR data.  $C_T$ : Threshold cycle; SEM: Standard Error of means. Unpaired two-tailed T-test comparing  $\Delta C_T$  values in cancer cell lines to  $\Delta C_T$  values in IOSE cells. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

	$C_T$ (SEM)	$\Delta C_T$ (SEM)	$\Delta\Delta C_T$ (SEM)
SKOV-3			
GAPDH	17.70 (0.20)		
RGS2	29.05 (0.15)	11.35 (0.05)	*0.35 (4.7e-7)
RGS4	37.25 (0.45)	19.55 (0.25)	***16.13 (0.27)
RGS6	34.35 (0.10)	16.65 (0.30)	*-2.4 (0.7)
RGS14	N.D.	N.A.	N.A.
RGS19	25.83 (0.22)	8.125 (0.425)	-1.2 (0.200)
CAOV-3			
GAPDH	15.05 (0.70)		
RGS2	27.13 (0.37)	12.08 (3.32)	1.075 (0.275)
RGS4	N.D.	N.A.	N.A.
RGS6	23.3 (0.65)	8.25 (0.05)	** -10.85 (0.45)
RGS14	41.70 (1.25)	26.65 (1.95)	1.175 (0.325)
RGS19	24.28 (0.22)	9.22 (0.47)	-1.000 (0.25)
OVCAR-3			
GAPDH	16.35 (0.10)		
RGS2	28.33 (0.02)	11.98 (0.075)	**0.975 (0.25)
RGS4	32.73 (0.02)	16.38 (0.07)	***12.95 (0.50)
RGS6	25.18 (0.42)	8.82 (0.32)	** -10.28 (0.70)
RGS14	44.05 (0.65)	27.70 (0.75)	2.22 (1.52)
RGS19	25.70 (0.45)	9.35 (0.55)	0.025 (0.325)
IOSE			
GAPDH	16.35 (0.30)		
RGS2	27.35 (0.25)	11.00 (0.05)	
RGS4	19.78 (0.78)	3.425 (0.035)	
RGS6	35.45 (0.70)	19.10 (0.40)	
RS14	41.83 (1.95)	25.48 (2.275)	
RGS19	25.68 (0.07)	9.32 (0.22)	

The quantitative results are consistent with the results described above. We found that RGS19 was detected at similar levels in each cell line, relative to GAPDH levels. RGS14 was detected in IOSE, Caov-3, and OVCAR-3 cells, albeit at very low levels, with no significant differences between expression levels, and was not detected in SKOV-3 cells. It is noteworthy that the  $C_T$  values obtained for RGS14 were greater than 40, beyond the manufacturer's suggested reliable range of detection for Taqman expression reagents; however, negative controls lacking reverse transcriptase enzymes or lacking template altogether consistently failed to cross threshold fluorescence signal after 50 cycles of amplification.

Significant differences in the relative expression levels of RGS2, RGS4, and RGS6 transcripts were observed. RGS2 transcript was expressed at slightly but

significantly lower levels in SKOV-3 and OVCAR-3 cells as compared to IOSE cells. Expression was also consistently lower in Caov-3 cells, but the data did not meet criteria for statistical significance due to greater variability (Fig. 2A). Differences in expression of RGS4 and RGS6 were much more dramatic. RGS4 transcript expression was reduced 5000-fold or greater in each of the ovarian cancer cell lines as compared to expression in IOSE cells. Fold changes in expression could not be calculated for RGS4 in Caov-3 cells because RGS4 was not detected in Caov-3 RNA (consistent with the qualitative RT-PCR results, Fig. 1), denoted by the white bar in Fig. 2B. RGS6 displayed the opposite expression pattern, being expressed at higher levels in cancer cell lines relative to IOSE cells. RGS6 transcript was expressed in SKOV-3 cells at levels approximately 5-fold higher than in IOSE cells, and more than 1000-fold higher in Caov-3 and OVCAR-3 cells as compared to IOSE cells, also consistent with qualitative results shown above.

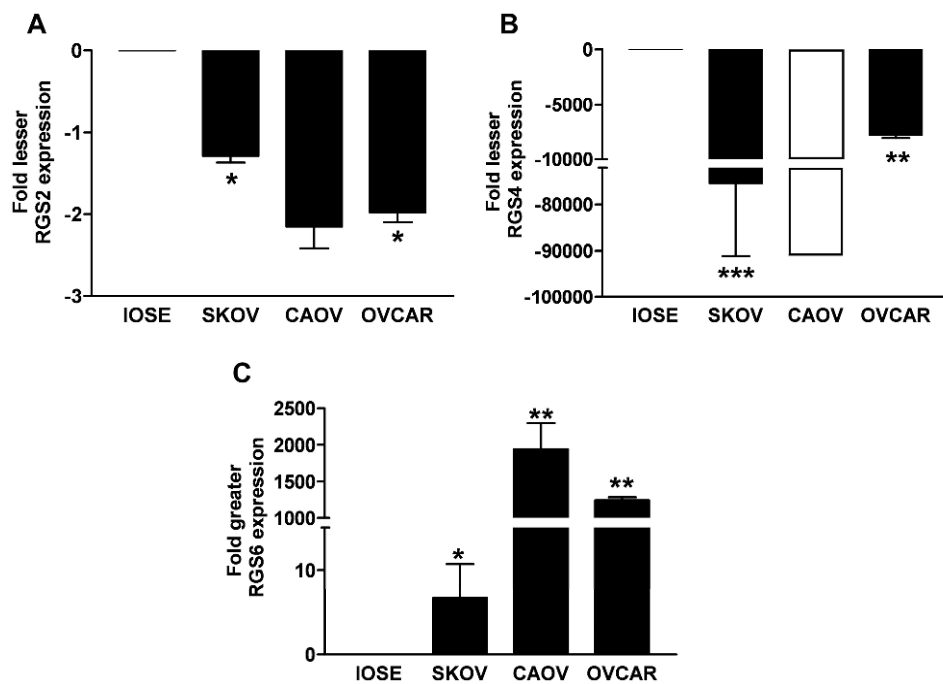


Fig. 2. Expression of RGS2, RGS4, and RGS6 in ovarian cancer cell lines relative to expression in IOSE cells. RNA was prepared from IOSE, SKOV-3, Caov-3, and OVCAR-3 cell lines and real time RT-PCR reactions were performed using Taqman Gene Expression Assays (Applied Biosystems). Positive fold changes in expression were calculated according to the  $2^{-\Delta\Delta CT}$  method, and negative fold changes were calculated as  $-1/2^{-\Delta\Delta CT}$ . Results represent cumulative data from two independent experiments.

**RGS regulation of individual exogenously expressed LPA receptors**

We next sought to determine the ability of RGS6, RGS2, and RGS19/GAIP to affect the strength of LPA signalling cascades. LPA's effects on  $G_q$  and  $G_i$  pathways are well documented in multiple systems, and each of the three classic LPA receptors LPA1, LPA2, and LPA3 are known to couple to both  $G_i$  and  $G_q$  pathways [9, 19-22]. We assessed LPA receptor signalling and regulation by RGS proteins in an assay system that reflects activation of both of these G-protein pathways: inositol phosphate production by phospholipase C (PLC) enzymes. Phospholipase C can be activated directly by  $G\alpha_q$  subunits [23], or by  $G\beta\gamma$  dimers downstream of  $G_i$  coupled receptors [24].

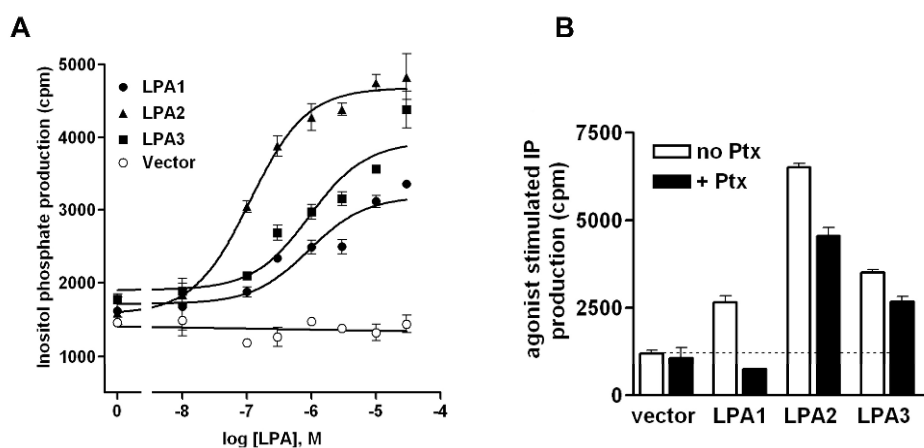


Fig. 3. Signaling by exogenously expressed LPA receptors in COS-7 cells. A – LPA receptor activation of inositol phosphate accumulation. LPA dose response curves were determined in inositol phosphate assays in COS-7 cells 48 hours after transient transfection with 100 ng/well of vector (○), LPA1 (●), LPA2 (▲), or LPA3 (■) in 24-well dishes. Cells were labeled with  $^3$ H myo-inositol and incubated with various concentrations of LPA in the presence of lithium chloride for 30 minutes. Inositol phosphates were isolated by ion exchange chromatography and quantitated with scintillation counting. B – LPA receptor sensitivity to pertussis toxin. Cells exogenously expressing LPA1, LPA2, or LPA3 were treated with 200 ng/ml of pertussis toxin (■) or vehicle (□) for 18 hours, and cells were assayed for inositol phosphate production in the presence of 30  $\mu$ M LPA. Basal values obtained in the absence of LPA (vehicle controls) have been subtracted from these data. Data shown are representative of three independent experiments.

First we determined the effect of the RGS proteins on each of the classic Edg family LPA receptors individually expressed in Cos-7 cells, which allow high levels of expression of multiple exogenous proteins via transient transfection. We first characterized LPA-stimulated inositol phosphate production in COS-7 cells expressing each receptor. Although COS-7 cells are known to contain LPA receptors [25], in the absence of transfected receptor, we observed only a modest, inconsistent increase in inositol phosphate production in response to

LPA (Fig. 3A). Expression of RGS proteins alone did not significantly alter this endogenous COS-7 LPA response (data not shown). However, transfection of LPA1, LPA2, or LPA3 imparted robust dose-dependent LPA-stimulated inositol phosphate accumulation, with  $EC_{50}$  values of approximately 0.9-1  $\mu$ M for both LPA1 and LPA3 expressing cells, and approximately 0.1  $\mu$ M for LPA2 expressing cells (Fig. 3A). To compare the G-proteins mediating the inositol phosphate response from the three LPA receptors, we treated cells with the  $G_i$  specific inhibitor pertussis toxin (Ptx). LPA stimulated inositol phosphate production downstream of LPA1 was completely inhibited by Ptx, suggesting that in this system LPA1 activation of PLC enzymes is mediated by  $G_i/o$  type G-proteins. LPA2 and LPA3 dependent activation of PLC activity was inhibited by Ptx by approximately 30% each, suggesting that these receptors predominately utilize Gq-like Ptx insensitive G-proteins to activate PLC in this system (Fig. 3B).

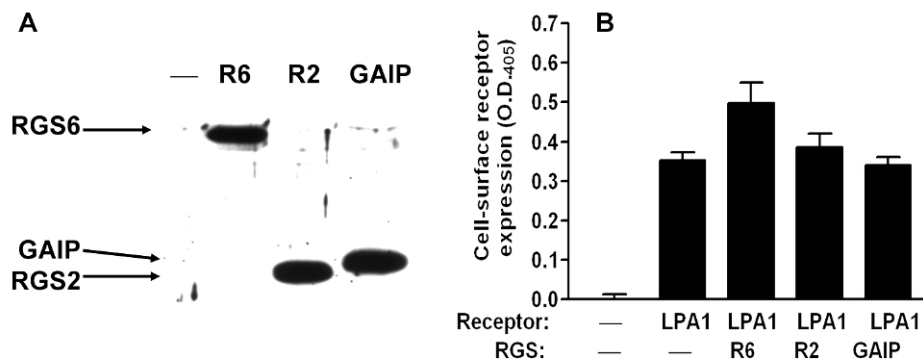


Fig. 4. Co-expression of exogenous RGS proteins and LPA receptors. COS-7 cells were transfected with LPA1 receptor alone or along with RGS6, RGS2, or RGS19/GAIP in parallel with the inositol phosphate assay to compare RGS expression and receptor cell-surface expression. A – Expression of RGS proteins in COS-7 cells. Cell lysates were harvested 48 hours after transfection and separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-HA antibody and HRP-conjugated secondary antibody. B – Effect of RGS expression on LPA receptor cell surface expression in COS-7 cells. COS-7 cells were plated in 96-well plates and transfected with 100 ng total plasmid DNA/well at the same proportions as in the inositol phosphate assay. Cells were fixed and incubated with anti-HA antibody and detected as described. Similar results were obtained in experiments with LPA2 and LPA3 co-transfections. Data are representative of at least three independent experiments.

To evaluate the effect of RGS function on LPA signalling pathways downstream of each receptor, we compared activation of inositol phosphate production by LPA in cells expressing exogenous LPA receptor alone versus cells expressing LPA receptor along with RGS6, RGS2, or RGS19/GAIP. Each of the RGS proteins was N-terminally tagged with a 3X HA epitope, so expression levels of the different proteins could be compared directly. The amount of each

expression construct required to yield comparable protein expression levels following transient transfection was determined empirically. Each of the RGS proteins was expressed at similarly high levels in COS-7 cells following transient transfection of 50 ng of plasmid DNA encoding RGS19/GAIP, 400 ng of plasmid DNA encoding RGS2 and 200 ng of plasmid encoding RGS6 per well in a 24-well dish (Fig. 4A). [RGS6 protein is found in tissues as a heterodimer with its binding partner G $\beta$ <sub>5</sub>, which is required for its stable expression [26]. Thus, G $\beta$ <sub>5</sub> was cotransfected (200 ng plasmid DNA/well) with RGS6 in all experiments]. In addition to the predicted inhibitory activity on G-proteins, it is possible that RGS proteins could inhibit exogenous LPA receptor signalling by lowering the amount of LPA receptor expressed on the cell surface. To confirm that any observed RGS effects on LPA signalling were mediated downstream of the receptor, the LPA receptors were each N-terminally

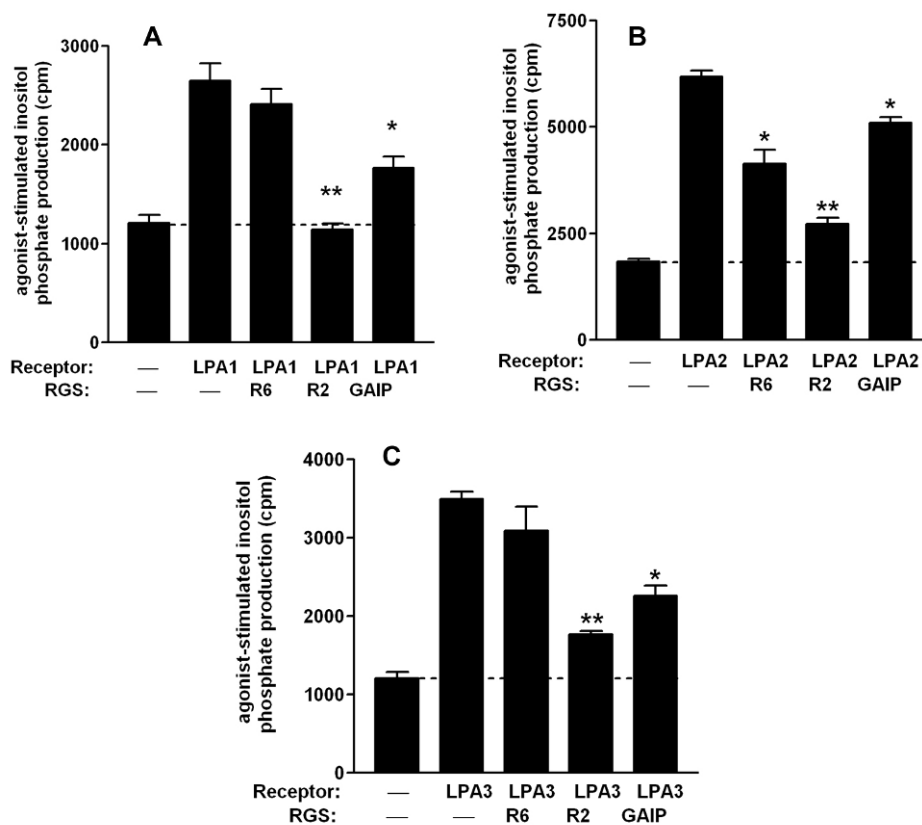


Fig. 5. The effect of RGS expression on LPA signaling activity in the inositol phosphate assay. Cos-7 cells were transfected with 100 ng of LPA1 (A), LPA2 (B), or LPA3 (C) and 400 ng of vector, 200 ng/200 ng RGS6/G $\beta$ <sub>5</sub>, 400 ng RGS2, or 50 ng GAIP. LPA responses were determined in inositol phosphate assays using vehicle or 10  $\mu$ M LPA as described in Methods. Data are representative of three independent experiments.

epitope tagged to monitor their cell-surface expression. Whole-cell ELISAs were performed with each experiment to ensure that co-expression of RGS proteins did not inhibit LPA receptor expression. In each case, co-expression of RGS proteins either had no effect or slightly increased LPA receptor cell-surface expression, but did not lower LPA receptor expression (Fig. 4B). Expression of RGS proteins alone did not affect ELISA signal (data not shown).

LPA-stimulated inositol phosphate signalling in cells expressing LPA1 receptors was completely inhibited by co-expression of RGS2 and partially inhibited by similar expression levels of RGS19/GAIP, but RGS6 expression had no significant effect on activity (Fig. 5A). RGS2, RGS6, and RGS19/GAIP each significantly inhibited LPA signalling mediated by LPA2 receptor expression, with RGS2 again exerting the strongest effect (Fig. 5B). Finally, in LPA3 expressing cells, RGS6 did not significantly inhibit signalling, while RGS2 and RGS19/GAIP partially inhibited the LPA3 receptor mediated response (Fig. 5C).

#### **Effect of RGS proteins on endogenous LPA receptor signalling in ovarian cancer cells**

We next determined if overexpression of individual RGS proteins could also affect signalling mediated by LPA receptors endogenously expressed in SKOV-3 ovarian cancer cells. SKOV-3 cells displayed a robust LPA stimulated inositol phosphate response with an  $EC_{50}$  of approximately 500 nM (Fig. 6D) which was inhibited by pertussis toxin treatment by approximately 70% (Fig. 6B). Expression of RGS2 in SKOV-3 cells strongly inhibited the LPA stimulated inositol phosphate response, while expression of RGS6 and RGS19/GAIP did not (Fig. 6C). LPA dose response curves in SKOV-3 cells transfected with either vector or RGS2 demonstrated that expression of RGS2 in these cells lowered the maximal effect of LPA by approximately 50%, and lowered the potency of LPA by nearly an order of magnitude (Fig. 6D).

We further tested the ability of RGS2, RGS6, and RGS19/GAIP to regulate LPA signalling in Caov-3 cancer cells. Caov-3 cells displayed an LPA-stimulated inositol phosphate response, although with distinctly lower potency ( $EC_{50}$  of approximately 10  $\mu$ M, Fig. 7D), and with lower sensitivity to pertussis toxin treatment (40% inhibition, Fig. 7B) than in SKOV-3 cells, suggesting that the response in these cells may be mediated by different receptors and/or G-proteins. In Caov-3 cells, significant inhibition of LPA signalling was observed consistently with RGS2 expression (Fig. 7C), although the degree of inhibition varied between experiments, reflecting the observation that LPA responses fall off sharply at high LPA doses with expression of exogenous RGS2 (Fig. 7D). Significant and consistent inhibition of LPA signalling was observed in the presence of RGS19/GAIP expression in CAOV-3 cells (Fig. 7C), and the dose response to LPA shows 50% lower maximal efficacy in the presence of expressed GAIP, with no significant change in LPA potency (Fig. 7D). Again, RGS6 did not inhibit LPA signalling in CAOV-3 cells.

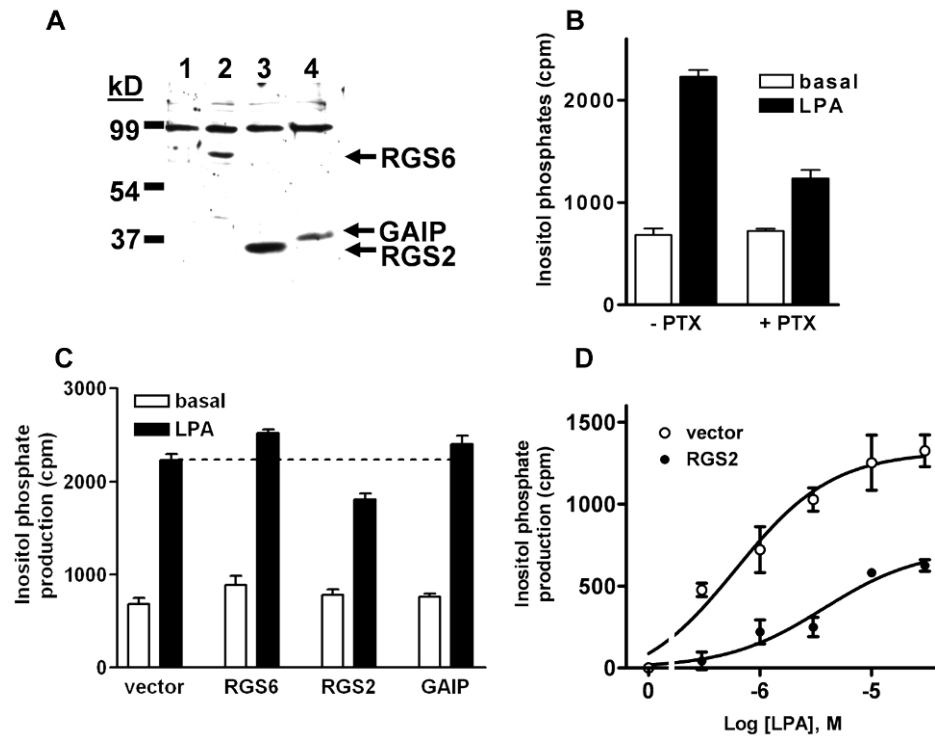


Fig. 6. RGS regulation of LPA signalling in SKOV-3 cells. A – RGS expression in SKOV-3 cells. Cells were plated in 24-well dishes and transfected with: 1) vector; 2) RGS6 (and G $\beta_3$ ) plasmid DNA; 3) RGS2 plasmid DNA; or 4) RGS19/GAIP plasmid DNA in parallel with inositol phosphate assays. Cell lysates were harvested 48 hours after transfection and separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-HA antibody and HRP-conjugated secondary antibody. B – Pertussis toxin sensitivity of LPA-stimulated inositol phosphate production in SKOV-3 cells. SKOV-3 cells were treated with 200 ng/ml of pertussis toxin or vehicle for 18 hours, and cells were assayed for inositol phosphate production in the presence of vehicle or 30  $\mu$ M LPA. C – Effect of RGS protein expression on LPA signaling in SKOV-3 cells. SKOV-3 cells were transfected with vector, RGS6, RGS2, or RGS19/GAIP. LPA responses were determined in inositol phosphate assays using vehicle or 10  $\mu$ M LPA. D – RGS2 decreases the efficacy of LPA signaling in SKOV-3 cells. SKOV-3 cells were transfected with 400 ng of vector ( $\circ$ ) or 400 ng RGS2 ( $\bullet$ ). LPA responses were determined in inositol phosphate assays using vehicle or various concentrations of LPA. Data are representative of three independent experiments.

## DISCUSSION

In this study, several RGS transcripts were detected in SKOV-3 cells which could account for our recent report that G-protein sensitivity to RGS regulation resulted in diminished LPA signalling capacity in SKOV-3 cells [14]. Further,

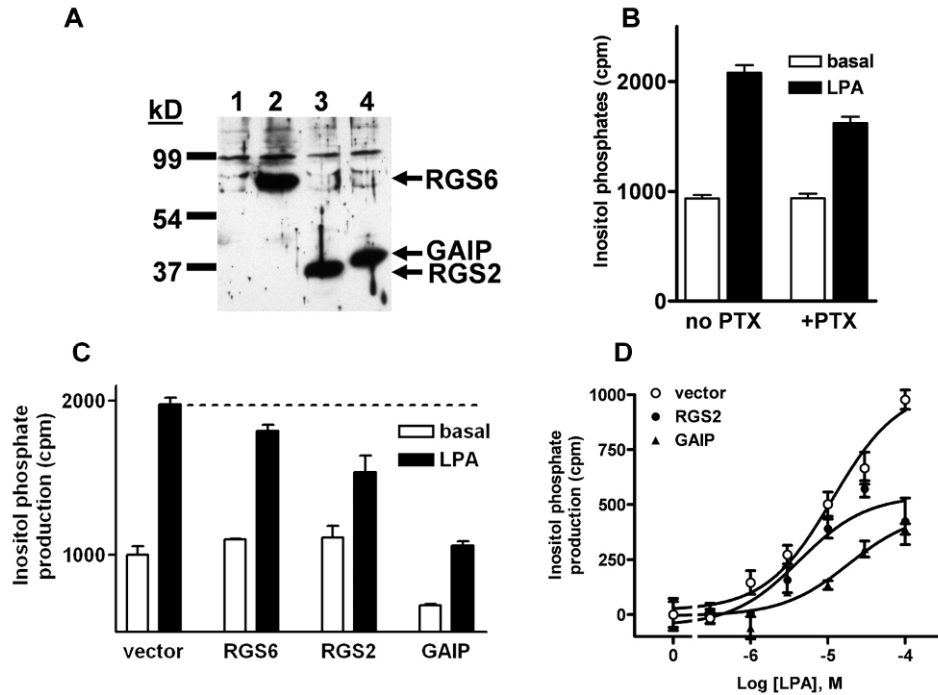


Fig. 7. RGS regulation of LPA signalling in Caov-3 cells. A – RGS expression in Caov-3 cells. Cells were plated in 24-well dishes and transfected with: 1) vector; 2) RGS6 (and  $G\beta_5$ ) plasmid DNA; 3) RGS2 plasmid DNA; or 4) RGS19/GAIP plasmid DNA in parallel with inositol phosphate assays. Cell lysates were harvested 48 hours after transfection and separated using SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-HA antibody and HRP-conjugated secondary antibody. B – Pertussis toxin sensitivity of LPA-stimulated inositol phosphate production in Caov-3 cells. Caov-3 cells were treated with 200 ng/ml of pertussis toxin or vehicle for 18 hours, and cells were assayed for inositol phosphate production in the presence of vehicle or 30  $\mu$ M LPA. C – Effect of RGS protein expression on LPA signaling in Caov-3 cells. Caov-3 cells were transfected with vector, RGS6, RGS2, or RGS19/GAIP. LPA responses were determined in inositol phosphate assays using vehicle or 10  $\mu$ M LPA. D – RGS2 and GAIP decrease the efficacy of LPA signaling in Caov-3 cells. Caov-3 cells were transfected with vector ( $\circ$ ), RGS2 ( $\bullet$ ) or GAIP ( $\blacktriangle$ ). LPA responses were determined in inositol phosphate assays using vehicle or various concentrations of LPA as described in Methods.

RGS transcripts were found to be widely expressed in additional ovarian non-transformed and cancer cell lines, and several are differentially expressed among these cell lines. Most notably, RGS4 transcript is expressed at much higher levels in non-transformed ovarian cancer cell than in cancer-derived cell lines, while RGS6 transcript is expressed at much higher levels in ovarian cancer cell lines than in non-transformed ovarian cells.

We chose three representative RGS proteins – RGS2, RGS6, and RGS19/GAIP – to test further for functional regulation of LPA signalling pathways in



a defined receptor expression system and in two ovarian cancer cell lines expressing endogenous LPA receptors. RGS2, RGS6 and RGS19/GAIP were each detected in all three of the ovarian cancer cell lines tested. RGS2 and RGS19/GAIP were expressed at similar levels in each cancer cell lines tested (although RGS2 was detected at significantly higher levels in IOSE non-transformed cells), while RGS6 transcript was expressed at much higher levels in Caov-3 and OVCAR-3 than in SKOV-3 cells. RGS2, structurally one of the simplest RGS proteins, is widely expressed, particularly in cardiac tissues, and has been detected in ovary where its expression is increased by stimulation with luteinizing hormone [27]. RGS2 is known to selectively accelerate the GTPase activity of  $G_q$  *in vitro* [28], although  $G_i$  subunit GAP activity has also been reported in certain systems [29-31]. In contrast, RGS19/GAIP, which is widely expressed and found at highest levels in lung, heart and placenta [32], can act as a GAP for both  $G_i$  and  $G_q$ , but is strongly selective for  $G_i$  G-proteins *in vivo* [33-35]. RGS6 is also a  $G_{i/o}$ -specific RGS protein [36]. Although RGS6 is expressed at highest levels in heart and brain, RGS6 transcript has been detected in multiple other tissues including ovary [37].

To observe potential effect of RGS proteins on either  $G_i$  or  $G_q$  signalling, we measured LPA effects on PLC activity, which can be regulated by both  $G_i$  and  $G_q$  as described above. We found that each of these RGS proteins had the capacity to attenuate signalling from LPA receptors in an exogenous expression system, while only RGS2 and RGS19/GAIP significantly inhibited signalling by LPA receptors endogenously expressed in ovarian cancer cell lines. We first determined the role of RGS regulation in a defined overexpression system to determine the effects of RGS proteins on signalling by individual LPA receptors. LPA 1, 2, and 3 appeared to couple to different G-proteins to activate PLC in COS-7 cells: LPA 1 was coupled primarily to  $G_{i/o}$  type G-proteins, while LPA2 and LPA3 were primarily  $G_q$ -like Ptx insensitive G-proteins. Based on this observation we predicted that LPA1-mediated signalling would be most strongly regulated by RGS6 and RGS19/GAIP, which are  $G_{i/o}$  selective RGS proteins, while signalling mediated by LPA2 and LPA3 would be most highly regulated by RGS2. However, we observed inhibition of signalling from of all three receptors by RGS2 and RGS19/GAIP expression, while RGS6 selectively inhibited signalling from LPA2, but had no effect on signalling from either LPA1 or LPA3. While the non-selective regulation by RGS2 and RGS19 may be explained by a loss of specificity due to overexpression, the lack of effect of RGS6 on LPA signalling via LPA1 was unexpected. Future studies are required to define the mechanisms of receptor specificity.

RGS2 partially inhibited endogenous LPA signalling in both SKOV-3 and CAOV-3 ovarian cancer cell lines, although differences were observed in the effect of RGS2 expression on the dose response curve of LPA in the two cell types. In SKOV-3 cells, RGS2 expression appeared to result in an overall lowering of LPA potency and efficacy. In Caov-3 cells, however, LPA effects were only affected by RGS2 expression at high doses of LPA and may reflect

non-specific effects. While RGS19/GAIP had no effect on LPA signalling in SKOV-3 cells, we observed strong inhibition of LPA efficacy in Caov-3 cells. Our data do not suggest a significant effect of RGS6 expression on LPA signalling in either SKOV-3 or Caov-3 cells. Ptx inhibition data suggest that the LPA response in SKOV-3 cells is more Gi-dependent than in Caov-3. Surprisingly, the Gq-selective GAP RGS2 had a greater effect in SKOV-3 cells and the Gi-selective GAP RGS19 had a greater effect in CAOv-3 cells. While it is possible that distinct RGS proteins have greater regulatory effects in different cell lines due to specific interaction with unique signalling pathways coupled to LPA receptors in different cells, it is also possible that this reflects a loss of specificity due to overexpression.

While it is tempting to speculate that the observed effects of RSG2 on LPA stimulated inositol phosphate production in SKOV-3 cells may have contributed to the recently reported regulation of LPA stimulated cellular migration and inhibition of cAMP accumulation by endogenous RGS proteins in SKOV-3 cells [14], such assignment is premature. While LPA stimulated cellular migration and inhibition of cAMP were completely dependent on Ptx sensitive Gi/o G-proteins, in the current study we assayed inositol phosphate accumulation, which was only partially Ptx sensitive and may have been mediated by both Ptx sensitive and Ptx insensitive G-proteins such as Gq. RGS2 preferentially targets Gq G-proteins, but has been reported to deactivate Gi/o G-proteins under certain conditions. Future siRNA knock-down studies should more specifically determine the role of individual RGS proteins on distinct LPA signalling pathways when expressed at endogenous levels.

In summary, our data support a possible role for RGS2 and RGS19 in the regulation of LPA signalling in ovarian cancer cells. In addition to LPA, the gonatropin Luteinizing hormone [38] and the chemokine Growth-Regulated Oncogene  $\alpha$  (GRO $\alpha$ ) [39] also regulate ovarian cell growth by activating heterotrimeric G-proteins. Thus, RGS proteins expressed in ovarian cancer cells may regulate these pathways as well. Further, we report dramatic differences in expression levels of RGS transcripts in commonly used Ovarian cancer model cell lines. SKOV-3, OVCAR-3, and Caov-3 are all derived from human ovarian adenocarcinomas, but vary with respect to metastatic potential and invasiveness [40]. Differences in the expression of signalling regulators between these cell lines may account for differences in cellular function. Future studies should determine if the differences in RGS expression among ovarian cell lines and tumor tissues contributes to variability in G-protein regulated pathways relevant to cancer progression.

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