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GS2 as a retinol transacylase and as a catalytic dyad independent regulator of retinylester accretion

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ABSTRACT

GS2 (PNPLA4; iPLAη) is the smallest member of the patatin-like family of phospholipases (PNPLA). It was initially identified by its ability to hydrolyze retinylesters (RE) in cell homogenates, and was later found to esterify retinol using a variety of acyl donors. In the present study we set out to determine its cellular function and examined its impact on RE status in 293T cells transfected with GS2, GS2-M1 (a non-translatable mutant of GS2) and empty vector, in fibroblasts isolated from normal and GS2-null donors and in SCC12b and in a somatic cell knock-out of GS2 (SCC12b-GS2$^{neo/-}$), that we generated by homologous recombination. At 50 nM medium retinol, GS2 had no significant impact on RE accumulation. However, at 2 μM retinol, GS2 promoted a 1.6- to 5-fold increase in RE accumulation. To verify role of GS2 as a catalyst, RE levels were measured in 293T transfected wild type GS2, catalytic dyad mutants devoid of enzymatic activity, or alanine substitution mutants spanning the entire GS2 sequence. Surprisingly, every GS2 mutant promoted RE accumulation. This activity was also observed in the GS2 paralogues and rat orthologue. The data demonstrate that within the context of the cell GS2 promotes RE accumulation and may do so either as a catalyst or as a regulatory protein that enhances RE formation catalyzed by other acyl transferases.

Retinoic acid (RA) is a transcription factor ligand for a family of heterodimeric nuclear transcription factors which together control the expression of several hundred genes in a tissue specific manner [1,2] and regulate epidermal homeostasis [3]. RA delivered outside of a narrow nM range leads to aberrant epidermal maturation. In target tissues, such as the epidermis, RA can be biosynthesized from retinol, which is available at μM serum concentrations bound to retinol binding protein (RBP) [4,5]. The availability of retinol for oxidation to RA is limited by retinol esterification with long chain fatty acid esters in reactions catalyzed by three enzyme activities. First is lecithin:retinol acyl transferase (LRAT) which uses the sn-1 position of phosphatidyl choline as acyl donor, has a low Km for retinol and utilizes both free retinol and retinol bound to cellular retinol binding protein (CRBP) [6,7]. Next is acyl CoA retinol:acyl transferase (ARAT) which comprises multiple enzymes that utilize fatty acyl CoA as acyl donor and which have high Km's for retinol. These enzymes do not utilize holo-CRBP. They may play a role in RE accretion in tissues not expressing LRAT and in circumstances where retinol levels greatly exceed CRBP levels (i.e., pharmacologic dosing) [7–9]. Lastly is GS2, an enzyme initially identified by its RE hydrolase activity, but which also transacylates retinol with a variety of acyl donors [10,11].

Retinylester hydrolysis is catalyzed by a variety of enzymes in different tissues. Secreted RE hydrolases are required required for the uptake of retinol from dietary RE and chylomicron remnants. Other enzymes such as the carboxylesterases, hormone-sensitive lipase (HSL) and GS2 [10,12–15] are used intracellularly to hydrolyze RE. Together these enzymes allow retinol mobilization, subsequent delivery to target tissues, and substrate retinol for oxidation to RA.

GS2 (PNPLA4; iPLAη) is the smallest member of the patatin-like family of phospholipases (PNPLAs) with homology to the N-termini of the PNPLA family [16]. The gene for GS2 is present in humans and rats, but is absent from the mouse genome. In the human genome, GS2 is found on the X-chromosome at Xp22.3 between the genes for steroid sulfatase (STS) and Kallman syndrome (KAL) [17]. Like other members of the patatin family, GS2 contains an essential Ser-Asp catalytic dyad comprising a Gly-X-Ser-X-Gly motif and an Asp-X-Gly/Ala motif [18–20]. Alanine substitution of the Ser or Asp in GS2, as in patatin itself, results in the loss of enzymatic activities [10,21]. GS2 has three paralogues which show high homology (55–58%) to GS2 in their N-terminal domains. These proteins include TTS2.2 (PNPLA2; adipose triglyceride lipase or ATGL; iPLAζ), adiponutrin (PNPLA3; ADPN; iPLAε) and GS2-like (PNPLA5). Its rat orthologue, rGS2, is 80% homologous [16]. However, these

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homologies do not predict functional similarities. Human GS2 and TTS2.2 appear more similar than human GS2 and rGS2. In cell homogenates, both human proteins hydrolyze RE (albeit TTS2.2 activity is modest), both hydrolyze triacylglycerides and generate 1,2-diacylglycerol and 1,3-diacylglycerol intermediates, and both esterify retinol using triglyceride as acyl donor. In contrast, rGS2 does not hydrolyze RE, and although it hydrolyzes triglycerides, rGS2 does not generate 1,3-diacylglycerol, and does not esterify retinol using triglycerides as acyl donors [10,11]. Non-epitope tagged adiponutrin and GS2-like protein are even less similar to GS2. These proteins have limited ability to hydrolyze RE or triglycerides and neither has been found to catalyze retinol esterification [11,22].

With the aim of determining the role of GS2 in vivo, we compared RE accumulation in GS2 knock-out and GS2 over-expressing cell lines. Catalytic potential was distinguished from in vivo function, by comparing retinol esterification and RE accumulation in homogenates and cell culture, respectively. We found that under retinol sufficiency (2 μM) GS2 promotes RE accretion. The data further support the notion that at least some of the GS2-driven RE accumulation does not require the catalytic dyad.

Materials and methods

Cell culture

293T cell growth and transfections were carried out as previously described [10,11]. SCC12b [23] was grown using 3T3 feeder cells [24] with media modifications [25]. Fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). Fibroblasts were obtained under approvals by the human use review boards at Stony Brook University, Stony Brook, NY, USA, and Innsbruck Medical Center, Innsbruck, Austria.

Western blot analyses

Western blots were carried out as previously described [26] using a 1:200 dilution of rabbit anti-human GS2 as primary.

Transacylase and RE hydrolase assays

Retinol esterification (transacylation) and RE hydrolysis was measured in cell lysates (200 μL) that were prepared as previously described [10]. To measure transacylation, the lysates were incubated with 5 μM retinol for one hour at 37 °C after which retinoids were extracted and resolved by HPLC [10,25]. To measure RE hydrolysis the lysates were incubated with 33 μM retinyl palmitate as substrate for one hour at 37 °C after which retinoids were extracted and resolved by HPLC [10,25]. Absorbance at 326 nm was used to detect retinoids and spectral analyses were carried out to verify product identity.

Real-time PCR

Total RNA was isolated from cultured cells with RNeasy Mini kit (Qiagen, Valencia, CA) as described by the manufacturer. The concentration of RNA was determined by measuring OD_{260}. The quality of the RNA preparations was assessed by the ratio of OD260/280 (>1.9) and by the intact 28S and 18S ribosomal RNA bands observed on denaturing agarose gels following electrophoresis. For mRNA quantification, 5 μg of total RNA was separately reverse transcribed with 200 U Superscript II and 250 ng random primers (Invitrogen, Carlsbad, CA) in 20 μL mixtures of reverse transcription as recommended by the manufacturer. The cDNA then was diluted to 300 μl with 1 mM Tris–HCl and 0.1 mM EDTA, pH 8.0. Real-time PCR was performed in triplicate with 4 pmol amplifying primers and 8 μl cDNA in a final volume of 20 μL using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and the DNA Engine Opticon system (MJ Research, Waltham, MA). To quantify results, standard curves for real-time PCR were made using the following amounts of each cDNA: 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} pmol. Real-time PCR was carried out and analyzed with the program in the MJ Research Opticon System, a service provided by the DNA sequencing facility of SUNY at Stony Brook. The primer sequences are in Supplementary Table 1. All primer sets span at least one intron of their respective genes to avoid potential interference by DNA contamination of RNA samples.

Screening of Recessive X-Linked Ichthyosis (RXLI) patients for GS2 deletion

DNA from peripheral blood samples of each of 8 patients with RXLI due to STS deficiency was isolated and screened for GS2 deletion by PCR using markers on the X-chromosome: ZBED1 (2.4 M), NLGN4X (5.8 M), GS2 (7.7 M), Kal1 (8.4 M) and Fam9B (8.8 M). The primer sequences used are listed in Supplementary Table 2.

Isolation of GS2-null cells from skin biopsies

Eight mm punch biopsies were washed six times in Ca–Mg free phosphate buffered saline, pH 7.4 (PBS) and incubated overnight at 4 °C in DMEM supplemented with 2.5 mg/ml Dispase II (Boehinger Mannheim). Epidermis was then removed from the underlying stroma. To obtain dermal fibroblasts, the dermis was incubated with 20 mM HEPES, pH 7.2, supplemented with 130 mM sodium chloride, 10 mM Ca–acetate, and 3 mg/ml collagenase II (Boehringer Mannheim). After 45–60 min at 37 °C, the tissue was incubated with PBS containing 0.05% trypsin and 0.01% EDTA until the tissue dispersed. Cells were then harvested by centrifugation at 800*g*. Fibroblasts were grown in DMEM containing 10% fetal bovine serum (HyClone, Logan, Utah). Fibroblasts from the subcutaneous fat were obtained from explant culture using DMEM with 10% fetal bovine serum. Confirmation of the GS2 deletion was carried out by PCR using DNA isolated from the dermal fibroblasts.

Generation of GS2-null cells

To construct the targeting vector, human genomic DNA was isolated from 293T cells with the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The 2.5 kb 5′ arm containing exon 1 and a 4.5 kb 3′ arm containing exon 3 and 4 were then amplified by PCR with primers P1/P2 and P8/9, respectively, using High Fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA). The targeting vector was constructed by inserting the 5′ arm at SalI site and 3′ arm at BamHI(filled)/EcoRI sites in plasmid pKSloxpNT, generating pGS2KO. The sequence and orientation of insertions were confirmed by DNA sequencing and restriction mapping.

For experiments 50–70% confluent cultures of SCC12b were incubated with 0.25% trypsin in 0.53 mM EDTA at 37 °C for 5 min after which trypsin was neutralized with 5% FBS. Cells were collected by a 5 min centrifugation at 800*g*. Prior to electroporation, cells were washed once with PBS and resuspended at 2×10^7 cells/ml in PBS. 100 μg of pGS2KO vector DNA, linearized by XhoI digestion, ethanol precipitated and resuspended in 100 μl PBS, were mixed with 0.9 ml of the SCC12b cell suspension. The suspension was subjected to a 250 V, 500 μF pulse from a Gene Pulser (Bio-Rad Labs., Hercules, CA). After 5 min on ice, the cells were plated into 4 T-180 flasks. Selection with G418 (800 μg/mL) and ganciclovir (2 μM) (Sigma–Aldrich, St. Louis, MO) were started 48 h after the electroporation and medium was changed every 3 days