Hypoxia Inducible Lipid Droplet Associated protein inhibits Adipose Triglyceride Lipase

Krishna M. Padmanabha Das¹, Lisa Wechselberger¹*, Márton Liziczai¹, Montserrat De la Rosa Rodriguez², Gernot F. Grabner¹, Christoph Heier¹, Roland Viertlmayr¹, Claudia Radler¹, Jörg Lichtenegger¹, Robert Zimmermann¹,³, Jan Willem Borst⁴, Rudolf Zechner¹,³, Sander Kersten², Monika Oberer¹,³§

1 Institute of Molecular Biosciences, University of Graz, 8010 Graz, Austria
2 Division of Human Nutrition, Wageningen University, Wageningen, Netherlands
3 BioTechMed-Graz, Mozartgasse 12/I, 8010 Graz, Austria
4 Laboratory of Biochemistry and Microspectroscopy Research Facility, Wageningen University, Wageningen, Netherlands

* contributed equally
§ Corresponding author. Institute of Molecular Biosciences, University of Graz, 8010 Graz, Austria

Phone: +43 316 380 5483 Fax: +43 316 380 9850 Email: m.oberer@uni-graz.at

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Abbreviations: ATGL, adipose triglyceride lipase; HILPDA, Hypoxia inducible lipid droplet associated protein; Hig-2, Hypoxia inducible gene-2; G0S2, G0/G1 switch gene 2; LDs, lipid droplets; HSL, hormone sensitive lipase; MGL, monoacylglycerol lipase; CGI-58, comparative gene identification-58; IPTG, isopropyl β-D-1-thiogalactopyranoside; DPC, n-dodecylphosphocholine; MST, MicroScale Thermophoresis; SDS, sodium dodecyl sulfate, TAG, triacylglycerol, TGH, triacylglycerol hydrolase.
Abstract

Elaborate control mechanisms of intracellular triacylglycerol (TAG) breakdown are critically involved in the maintenance of energy homeostasis. Hypoxia inducible lipid droplet associated protein (HILPDA)/Hypoxia inducible gene-2 (Hig-2) has been shown to affect intracellular TAG levels, yet, the underlying molecular mechanisms are unclear. Here, we show that HILPDA inhibits adipose triglyceride lipase (ATGL), the enzyme catalyzing the first step of intracellular TAG hydrolysis. HILPDA shares structural similarity with G0/G1 switch gene 2 (G0S2), an established inhibitor of ATGL. HILPDA inhibits ATGL activity in a dose-dependent manner with an IC₅₀ value of ~2 µM. ATGL inhibition depends on the direct physical interaction of both proteins and involves the N-terminal, hydrophobic region of HILPDA and the N-terminal, patatin-domain containing segment of ATGL. Finally, confocal microscopy combined with Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) analysis indicated that HILPDA and ATGL colocalize and physically interact intracellularly. These findings provide a rational biochemical explanation for the tissue-specific increased TAG accumulation in HILPDA overexpressing transgenic mouse models.

Keywords: ATGL, HILPDA, HIG-2, Intracellular lipolysis, Adipocytes, Lipase, Lipid droplets, Lipolysis and fatty acid metabolism, Triglycerides
Introduction

In times of nutrient surplus, mammals store triacylglycerols (TAGs) in lipid droplets (LDs) of white adipose tissue. Under starving conditions, a process termed intracellular lipolysis hydrolyzes TAG stores leading to the release of fatty acids and glycerol, which are utilized in other tissues for energy production as well as lipid and membrane biosynthesis. Dysregulation of the coordinated processes of TAG synthesis and mobilization leads to various pathologies including metabolic syndrome, atherosclerosis, (non-alcoholic) fatty liver disease, certain forms of cancer including cervical and liver cancer, and cancer associated cachexia (1,2).

Lipolysis involves the consecutive action of three major lipases called adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MGL). The activity of ATGL, which hydrolyzes TAGs into diacylglycerols and unesterified fatty acids, is modulated by numerous transcriptional and post-transcriptional regulatory processes including interaction with protein co-regulators (3). The co-activator protein comparative gene identification-58 (CGI-58, also termed alpha-beta hydrolase domain-containing 5, ABHD5) activates, whereas G0S2, the gene product of the G0/G1 switch gene 2, inhibits the TAG-hydrolyzing activity (4,5). In humans and mice, the ATGL inhibitor G0S2 is a 103 amino-acid protein with high expression levels in adipose tissues, bone marrow, skeletal muscle and liver (5–8). Direct protein-protein interaction of G0S2 with the patatin-domain within ATGL mediates the selective inhibition of enzyme activity (9,10). G0S2 is a peroxisome proliferator-activated receptor γ (PPARγ) target gene (8) and, additionally, highly upregulated during hypoxia (11). Tissue-specific overexpression or deletion of G0S2 in mice strongly affects TAG accumulation in various tissues (12).

Recent work identified another LD-associated protein that is structurally related to and similarly regulated as G0S2 named hypoxia-inducible lipid droplet-associated protein (HILPDA) (13–16). HILPDA comprises a 63 amino acid protein and is also known as Hypoxia-inducible protein 2 (HIG2). It was originally identified in cervical cancer cells upon hypoxic stress (17). Similar to G0S2, HILPDA expression is regulated by hypoxia-inducible factor-1, peroxisome proliferator-activated receptors (PPARs) and β-adrenergic stimulation (13,15,18). Highest expression levels are observed in brain,
endocrine tissues, muscle (skeletal muscle and heart), lung, liver, and adipose tissue (13,19,20). Additionally, HILPDA is abundantly expressed in many cancer tissues and atherosclerotic plaques (13,20).

The physiological function of HILPDA remains unclear. Its regulation by PPARs and localization on LDs suggested a role in neutral lipid metabolism (18). Consistent with this hypothesis, Hilpda gene deletion in mice resulted in loss of LD formation in macrophages, reduced fat pad weight and lower hepatic lipid levels, while overexpression caused a steatotic phenotype in hepatocytes (13,14,16,18). Whether HILPDA directly interferes with the enzymatic catabolism of TAGs remains controversial (10-11). Considering the structural similarities between HILPDA and G0S2, we tested whether HILPDA acts as an inhibitor of ATGL enzyme activity. We show that HILPDA directly binds ATGL and inhibits its TAG hydrolase activity in vitro with an IC₅₀ value in the low µM range. The interaction of both proteins involves the patatin-domain containing region of ATGL and the N-terminal, predominantly hydrophobic region of HILPDA. Furthermore, we show a physical interaction between HILPDA and ATGL by Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) analysis.

Materials and Methods

Materials

If not stated otherwise, chemicals, antibiotics and buffers were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Carl Roth GmbH (Karlsruhe, Germany); columns for protein purification from GE Healthcare Life Sciences (Chicago, IL, USA). [9, 10⁻³H] Triolein was obtained from PerkinElmer Life Sciences (Waltham, MA, USA). Triolein, phosphatidylcholine, phosphatidylinositol, 1(rac)-oleoylglycerol, oleoyl-CoA, and free glycerol detection reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Sequence Analysis

Amino acid sequences for HILPDA and G0S2 were retrieved from the UniProt knowledgebase (21), the sequence alignment and phylogram were generated with Clustal Omega (22). Structure prediction was carried out in Phyre2 (23), the figure displaying the predicted structure was generated using PyMOL (24).

Cloning of HILPDA, ATGL and HSL constructs

The cDNA clone for human HILPDA (hHILPDA) was obtained from Dharmacon, USA (Accession: BC112183.1, MGC: 138388). The expression cassette was cloned into a modified pSUMO vector (9) with a 6XHis tag and a TEV cleavage site, following the Gibson assembly cloning protocol (25) (New England BioLabs, Ipswich, MA, USA) using primers mentioned in Table 1. N-terminal truncations were generated following the Gibson assembly protocol, and C-terminal truncations were made using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs, Ipswich, MA, USA). The coding sequence of murine HSL was amplified by PCR from the pcDNA4/HisMaxA-mHSL construct described in (4) using the primers listed in Table 1. The PCR product was cloned into pECFP-N1 vector (Clonetech, Mountain View, California, USA) using the XhoI and SacII restriction sites. The coding sequence for mATGL encoding residues 1-288 and for superfolder (sf) GFP was amplified using primers described in Table 1. The resulting DNA fragments were cloned into the pST50 vector (26) following the Gibson assembly cloning protocol. The correctness of all generated inserts was verified by DNA sequencing.

Bacterial expression and purification of HILPDA and mATGL

The expression plasmids coding for 6XHis-smt3-TEV-hHILPDA or truncated variants were transformed into BL21-CodonPlus (DE3)-RIL competent cells (Stratagene, La Jolla, CA, USA), and the cultures were grown at 37 °C in Luria-Bertani (LB) medium containing kanamycin (50 µg/ml) to an OD$_{600}$ of 0.6. Cells were cooled down to 16 °C and induced with 0.5 mM IPTG. Following overnight induction, cells were harvested and lysed in lysis buffer (20 mM Tris/HCl, 300 mM NaCl, 0.05 % IGEPAL® CA-630 pH 7.8) by sonicating at 50% amplitude for 5 min (SONOPPLUS HD2070, Bandelin, Berlin, Germany). The soluble fraction was collected after centrifugation at 18,000 rpm for 40 minutes.
The cleared lysate was loaded to a pre-equilibrated HisTrap FF column, washed with 10 column volumes of wash buffer (20 mM Tris, 300 mM NaCl, 30 mM imidazole, 10% glycerol, pH 7.8) and eluted over a linear gradient against the same buffer, yet with 300 mM imidazole. Fractions containing the purified fusion protein was concentrated and subjected to gel filtration chromatography on a HiLoad 26/60 Superdex 200 preparatory column using SEC buffer (15 mM Na$_2$HPO$_4$, 5 mM KH$_2$PO$_4$, 300 mM NaCl, 1mM EDTA, 1 mM DTT, pH 6.8). The peak corresponding to the fusion protein was concentrated, quantified and used for triacylglycerol hydrolase (TGH) assay.

mATGL288 used for TGH assay and sf-GFP-mATGL288 used for MST measurements were expressed in ArcticExpress (DE3) Competent Cells (Agilent Technologies, Palo Alto, CA, USA) for 24h at 10°C. To purify the sf-GFP-mATGL_288 variant, the bacterial pellet was resuspended in ATGL lysis buffer (80 mM K$_2$HPO$_4$, 20 mM KH$_2$PO$_4$, 100 mM KCl, 10% glycerol, 1 mM TCEP, 10 mM ATP, 10 mM MgCl$_2$ and 1mg/ml n-dodecylphosphocholine (DPC), pH 7.5), lysed by sonication, and centrifuged. The supernatant was loaded on to pre-equilibrated Strep Trap HP column and washed with ATGL wash buffer (250 mM Tris/HCl pH 7.5, 500 mM KCl, 10% glycerol, 250 mM sucrose, 1 mM TCEP, 10 mM MgCl$_2$ and 10 mM ATP). The bound protein was eluted using ATGL elution buffer (100 mM Tris pH 7.5, 50 mM KCl, 10% glycerol, 250 mM sucrose, 1 mM TCEP, 1mM EDTA, and 10 mM desthiobiotin). The eluted protein was further purified using anion exchange chromatography over a ResourceQ column using the same buffer as above in a 0-500 mM KCl gradient. The peak fractions containing the ATGL variants, as judged by SDS-PAGE, were concentrated and loaded onto a Superdex 200 Increase 10/300 GL gel filtration column using ATGL SEC buffer (100 mM Tris/HCl pH 7.5, 100 mM KCl, 10% glycerol, 1mM TCEP, 1 mM EDTA, 1mg/ml DPC and 100 mM sucrose). The ATGL containing fractions were quantified and further used for MST experiments. Expression and purification of hG0S2 and mCGI-58 were done following previously described protocols (9,10).

Expression of recombinant proteins in HEK-293 cells and preparation of cell extracts for co-immunoprecipitation and enzyme activity assays

HEK293T cells were cultivated in DMEM (GIBCO, Invitrogen Corp., Carlsbad, USA), containing 10% FCS (Sigma-Aldrich, St. Louis, USA) at standard conditions (37 °C, 5% CO2, 95% humidified
atmosphere). Cells were transfected with 6 μg DNA (or 3 μg each in co-transfection experiments) complexed to Metafectene (Biontex GmbH, Munich, Germany) in serum-free DMEM. After 4 h, the medium was replaced by DMEM supplemented with 10% FCS. For co-immunoprecipitation experiments, cells were washed 3 times with Phosphate Buffer Saline (PBS), collected using a cell scraper, harvested by brief centrifugation, washed twice with PBS, and disrupted in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40). Cells were vortexed, incubated on ice for 30 minutes, and pressed 5 times through a 23G needle using a 1ml syringe for mechanical lysis. Lysed cells were centrifuged for 15 min, at 20 000 x g at 4°C and the supernatants were used for immunoprecipitation experiments. Protein samples for enzyme activity assays were prepared by sonication of transfected cells in 0.25 M sucrose, 1 mM EDTA and 1 mM DTT supplemented with 20 μg/ml leupeptin, 2 μg/ml antipain and 1 μg/ml pepstatin. Perinuclear supernatants were prepared by centrifugation at 1,000 x g for 10 min and were used for enzyme activity assays.

**Determination of protein concentration**

Protein concentration of cell lysates and purified proteins was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories Inc. Hercules, USA) according to the manufacturer’s instruction. BSA (Thermo Fisher Scientific Inc, Waltham, USA) was used for the generation of standard curves. Concentrations of some purified proteins were additionally obtained by the absorbance at 280 nm using the NanoDrop® ND-1000 spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

**Immunoblotting**

50 μg each of bacterial lysates containing hHILPDA and its truncated variants or ATGL lysates were loaded and run on a denaturing 12% SDS-PAGE gel and transferred to nitrocellulose membrane. Immunoblot was performed using mouse Penta His Antibody (Qiagen, 34660, Duesseldorf, Germany) or StrepMAB-Classic mouse monoclonal antibody (IBA Lifesciences, 2-1507-001, Goettingen, Germany) followed by ECL Anti-mouse IgG secondary antibody (GE Healthcare, Chicago, USA). The blot was developed using AmershamECL western blotting detection reagent (GE Healthcare, Chicago, USA).
In case of co-immunoprecipitation, immunoblot was performed using either monoclonal anti-Flag® M2-peroxidase HRP antibody (Sigma, St. Louis, USA) or Penta His Antibody (Qiagen, Duesseldorf, Germany) followed by mouse TruBlot® Anti-mouse Ig HRP secondary antibody (Rockland, Limerick, USA).

**Triacylglycerol hydrolase (TGH) assays and IC₅₀ determination**

TGH assays were performed essentially as described (27), yet on a smaller scale. In order to prepare bacterial lysates, harvested pellets were re-suspended in sucrose solution (250 mM sucrose, 1mM EDTA, 1mM DTT, 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) and disrupted by sonication as described earlier (9,28,29). 5 µg of purified HILPDA protein, 25 µg total protein of ATGL cell lysate and 1 µg of purified mCGI-58 were incubated with [³H] triolein containing substrate mix. 5 µg of purified hG0S2 instead of purified HILPDA were used in positive controls. To assess TAG hydrolase activity of murine HSL, the lipase was expressed as fusion protein (HSL-ECFP) in HEK-293 cells and perinuclear supernatants were prepared as an enzyme source as described above. 25 µg total protein of HSL cell lysate was incubated in the absence and presence of purified HILPDA. LPL was purified from bovine milk essentially as described (30) and 33 ng of LPL protein was used for the assay.

In order to prepare the TAG substrate, 1.67 mM triolein, 10 µCi/ml [9,10-³H] triolein, and 190 µM of phosphatidylcholine/phosphatidylinositol in a ratio of 3:1 were sonicated and the concentration was adjusted with the buffer. 25 µl of the respective protein preparation was incubated with 25 µl of [³H] triolein substrate and incubated at 37°C for one hour. The reaction was terminated by adding 650 µl of methanol/chloroform/heptane (10:9:7) and 200 µl of 100 mM potassium carbonate buffer, pH 10.5. After extraction and centrifugation, the radioactivity in 200 µl of the upper phase was determined by liquid scintillation counting. Dose response experiments of stimulated mATGL288 activity using increasing concentrations of hHILPDA (44 nM to 44 µM) were carried out to determine the half maximal inhibitory concentration (IC₅₀). Curve fitting to the data and calculation of the IC₅₀ value was implemented using GraphPad Prism 5 (GraphPad Software Inc., USA). The activities derived from TGH assays (measured from triplicates and at least two independent biological replicates) are represented as
mean ± S.D. Statistical significance was tested by the Student's unpaired two-tailed t test. Data are considered to be significantly different for p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***)..

**Monoacylglycerol hydrolase activity assay**

HILPDA was also analyzed for its ability to inhibit monoacylglycerol hydrolase activity of human MGL (hMGL) by following the assay protocol described earlier (32) using rac-1-3 oleoylglycerol as the substrate. A concentration of 5 nM of hMGL and HILPDA concentrations ranging from 2.5 nM to 250 nM were tested for the inhibition of hMGL.

**Co-immunoprecipitation**

Flag agarose beads were prepared by washing with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40) four times. 350 µg of HEK-293 cell lysate were incubated with 10 µl of Anti-Flag agarose beads (ANTI-FLAG® M2 Affinity Gel, Sigma-Aldrich St. Louis, MO, USA) over night at 4°C top in a sample shaker. Beads were washed four times with lysis buffer, and the beads were boiled in 30 µl of 2X SDS sample buffer (125 mM Tris-HCl pH 6.8, 5% SDS, 0.004% Bromophenol Blue, 10% β-mercaptoethanol, 20% glycerol). 15 µl of these samples were loaded onto 12% SDS PAGE gel and were subjected to immuno blotting as detailed earlier.

**Microscale thermophoresis**

Protein-protein interaction studies using MST was carried out on a Monolith NT.115 instrument (Nanotemper, Munich, Germany). Increasing concentrations (1.1 µM to 487 µM) of purified smt3 tagged HILPDA in buffer (20 mM phosphate buffer, 300 mM NaCl, 1 mM EDTA, 1 mM DTT pH 7.0) were titrated against constant amounts of Sf-GFP-mATGL_288 (0.01 µM). Samples were loaded into premium capillaries and measurements were performed in triplicates at 20% LED power and 40% MST power at room temperature. A constant concentration of 1% BSA was maintained in all the samples to avoid non-specific interactions. Data analysis was performed with Monolith software (Nanotemper, Munich, Germany) using both thermophoresis and T jump parameters.

**Cloning of mHILPDA and mATGL for fluorescence microscopy**
Plasmids of mHILPDA_sYFP2, mHILPDA_mCherry and mATGL_mTurquoise2 were constructed by fusing the full-length cDNA into pEGFP-N2 (Clontech, Mountain View, California, USA) and substituting the EGFP sequence by the sequence of the fluorescent proteins (FP) mCherry, sYFP2 or mTurquoise2. Briefly, RNA from mouse WAT and liver was reverse transcribed with First Strand cDNA synthesis kit (Thermo Scientific) and amplified with Phusion High fidelity DNA Polymerase (Thermo Scientific) using primers mentioned in Table 1. The PCR products were cloned into pEGFP-N2 vector using the XhoI and KpnI-HF (New England Biolabs Inc.) restriction sites. Afterwards, MAX Efficiency ® DH5α™ Competent Cells (Invitrogen) were transformed by heat-shock and grown in LB agar plates with kanamycin. The vector was isolated using Qiagen plasmid maxi kit (Qiagen) according to manufacturer instructions. The EGFP fragment was then excised from the pEGFP-N2 parent vector by enzyme digestion with KpnI-HF and NotI-HF. The vector was gel-purified with QIAquick Gel Extraction Kit (Qiagen) and the fragments of mCherry, sYFP2 or mTurquoise2 (31) were ligated using T4 DNA ligase (Thermo Scientific) into pEGFP-N2 vector which was digested with KpnI and NotI. The resulting constructs used are mHILPDA fused to either mCherry or sYFP2 and mTurquoise2 fused to mATGL.

Visualization of HILPDA and ATGL co-localization and interaction

Hepa 1-6 cells were cultured in DMEM (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Lonza) and 1% penicillin/streptomycin (Lonza) at standard conditions (37 °C, 5% CO2, 95% humidified atmosphere). Cells were seeded on a collagen coated (Ibidi, Martinsried, Germany) 8 well removable chamber (Ibidi, Martinsried, Germany) and grown for 24h before transfection. Transfections were performed with 700 ng of single or mixed plasmid DNA complexed to polyethylenimine (PEI) (Polyscience Inc., PA, USA) in serum-free DMEM. After 5 h, the medium was replaced by serum free DMEM supplemented with 1.5% fatty acid free BSA (Roche Applied Sciences) and 1.2 mM of oleic acid (OA) and palmitic acid mix (PA) in a 2:1 OA:PA ratio for 24h to promote lipid droplet formation. Cells were washed with PBS, fixed for 20 min with 4% formaldehyde and mounted with vectashield-H (Vector Laboratories, U.S.A). For colocalization analysis, Hepa 1-6 cells were transfected with
mATGL_mTurquoise2 and mHILPDA_mCherry. For FRET-FLIM analysis, Hepa 1-6 cells were transfected with mATGL_mTurquoise2 and mHILPDA_sYFP2 plasmids.

Colocalization imaging was performed on a Leica TCS SP5 X system equipped with a 63x 1.20 NA water-immersion objective lens. Images were acquired sequentially 1024x1024 pixel scans with pinhole set at 1 airy unit. mTurquoise2 was excited at 458 nm and fluorescence emission was detected using internal Hybrid (HyD) in a spectral window of 460-500 nm. mCherry was excited at 561 nm and detected using HyD in a spectral window of 588-655 nm. Images were process using Fiji. Briefly, a Gaussian Blur of 1 unit was applied; background was measured from the nucleus and subtracted.

FRET-FLIM was performed on a Leica TCS SP8 X confocal set-up including a picosecond Pulsed Diode Laser LDH-D-C-440 (PicoQuant), resulting in excitation pulses of 10 ps at a repetition of 40 MHz. Fluorescence emission was detected using HyD detectors with 100 ps time resolution and collected in a spectral window of 450-495 nm for the donor (mTurquoise2). The acceptor (sYFP2) was excited using a 40 MHz tunable supercontinuum laser at 514 nm and the fluorescence was detected from 540-600 nm. The signal output from the HyD detector was coupled to an external time-correlated single photon counting module (Becker&Hickl) for acquiring FLIM data. Typical images had 128 x 128 pixels (pixel size ± 300 nm), and 256 time channels per pixel with an acquisition time of 80 seconds per image.

From the time resolved fluorescence intensity images, the fluorescence decay curves were calculated for each pixel and fitted with a double-exponential decay model using the SPCImage v6.4 software (Becker & Hickl). Fitting was performed without fixing any parameters. FRET-FLIM analysis provided fluorescence intensity as well as false-colored fluorescence lifetime images. The raw data was subjected to the following criteria to analyze and omit false positive negatives in the fluorescence lifetime scoring: minimum photon count per pixel of 1200 photons, 2 component analysis, goodness of fit ($\chi^2<2$) and fluorescence lifetime range of 500–3500 ps. For data analysis, we set pixel binning at 1 to have sufficient number of photons per pixel required for accurate fluorescence lifetime analysis.
Results

HILPDA inhibits human and mouse ATGL under basal and CGI-58 stimulated conditions

The 103 amino acid protein G0S2 suppresses TAG catabolism via direct inhibition of the TAG-hydrolyzing activity of ATGL (5). In addition to similarities in expression, regulation, and intracellular localization, structural comparison of HILPDA and G0S2 revealed that the proteins also share amino acid sequence similarities (ca. 22% identity, 40% similarity). Accordingly, web portals for protein structure (23) predict similar secondary structures and similar spatial distributions of structural elements (Figure 1). To reveal potential functional similarities, we tested whether HILPDA is able to inhibit ATGL in vitro in a similar manner as G0S2. Previous studies identified the patatin-domain containing region of ATGL to be sufficient for catalysis, activation by CGI-58, and inhibition by G0S2 (10). Therefore, we hypothesized, that this region would also present the potential interface for HILPDA and used ATGL variants truncated after residue 288 (hATGL288, mATGL288) in our assays. To test the influence of HILPDA on ATGL, we first incubated E.coli cell lysates overexpressing a truncated yet fully active variant of hATGL with purified human HILPDA (Figure 2A). Purified smt3 and hG0S2 served as controls. As shown in Figure 2B, HILPDA as well as G0S2 inhibited the TAG activity of hATGL by ~90% while smt3 had no effect on enzyme activity. Similar as for hATGL, HILPDA also inhibited mATGL in the absence or presence of CGI-58 (Figure 2C). To investigate whether the inhibitory effect of HILPDA can be reproduced when recombinant proteins are expressed in mammalian cells, HEK293 lysates overexpressing hATGL and mATGL were tested for inhibition by HILPDA. Again, HILPDA inhibited both mouse and human ATGL in the presence or absence of CGI-58 (Figure 2D and 2E). The overexpression of ATGL in the lysates was verified by immunoblotting analysis (data not shown). When mATGL was co-expressed with HILPDA in HEK293 cells, mATGL activity was ~60% lower than in cells expressing only ATGL (Figure 2F). Again, HILPDA inhibited ATGL activity in the presence or absence of CGI-58. mATGL co-expression with hG0S2 reduced TAG hydrolase activity by ~90% suggesting a more potent ATGL inhibition effect of G0S2 than of HILPDA.

The N-terminal region of HILPDA is required and sufficient to inhibit ATGL activity
The N-terminal region of G0S2 has been identified to be essential for inhibition of ATGL (5,9). This region shares the highest sequence similarity with HILPDA (Figure 1A). To identify sequence motifs within the HILPDA polypeptide required for ATGL inhibition, we generated several C and N terminal truncations of the protein (Figure 3A). All constructs were expressed in E.coli, purified and subjected to an SDS-PAGE gel to estimate yield and purity (Figure 3B). Bacterial lysates overexpressing mATGL288 were used to test HILPDA variants for their ability to inhibit the TAG-hydrolyzing capacity of ATGL. C-terminal truncation variants of HILPDA (M1-E28, M1-M24 and M1-F20) inhibited ATGL to a similar extent as the full-length protein (Figure 3C). In contrast, the HILPDA peptide F20-M63 shows a reduced ability to inhibit ATGL, whereas the truncation E25-M63 lost the ability to inhibit ATGL activity (Figure 3C). These findings suggest that N-terminal segments in the HILPDA polypeptide, which share highest sequence conservation to G0S2, mediate the inhibition of ATGL activity.

**HILPDA directly binds to ATGL**

To assess whether HILPDA inhibition of ATGL requires direct protein-protein interaction, we performed co-immunoprecipitation experiments with HEK-293 cell lysates containing mATGL_FL_flag and GFP_HILPDA_His variants. A monoclonal anti-FLAG antibody bound to sepharose beads co-immunoprecipitated HILPDA along with ATGL (Figure 4A). Conversely, no HILPDA was immunoprecipitated when expressed alone or with empty control constructs arguing for a direct molecular interaction between ATGL and HILPDA.

To confirm this observation by an independent experimental approach, we investigated the HILPDA-ATGL interaction by MicroScale Thermophoresis (MST) with purified proteins (33). This technique depends on the directed motion of a molecule through a temperature gradient induced by an infrared laser. The monitored thermophoresis of a protein typically differs significantly from the thermophoresis of a protein–protein complex due to changes in size, charge and solvation energy. The technique allows the analysis of a fluorescence label. In our experiments, green fluorescent protein was N-terminally fused to ATGL to allow for analysis by fluorescence. MST analysis revealed ATGL-HILPDA binding
with a calculated $K_D$ of 247 µM (Figure 4B). No binding was observed when ATGL was titrated against smt3 protein alone (data not shown). The purified proteins used for MST analysis are shown in the inset.

**HILPDA and ATGL colocalize and physically interact intracellularly**

To further confirm the interaction between HILPDA and ATGL, we performed confocal microscopy and FRET-FLIM in Hepa 1-6 cells (Figure 5). We transfected Hepa 1-6 cells with mHILPDA_mCherry and mATGL_mTurquoise2 and observed that HILPDA partially colocalizes with ATGL (Figure 5A). Since confocal microscopy is diffraction limited to ~250 nm, our colocalization results do not directly prove that HILPDA and ATGL are physically interacting. To demonstrate protein interactions, we performed Förster resonance energy transfer (FRET) quantified by fluorescence lifetime imaging (FLIM). FRET is a process in which the excitation energy is transferred from a donor fluorophore to an acceptor chromophore through nonradiative dipole–dipole coupling when the fluorescent donor and acceptor molecules are in very close proximity (< 10 nm). FRET determined using FLIM is independent of protein concentration, but very sensitive for the local microenvironment of the fluorophores. In FRET-FLIM, the fluorescence lifetime of the donor molecule is reduced in the presence of an acceptor molecule nearby, since energy transfer to the acceptor will introduce an additional relaxation path from the excited to the ground state of the donor (34). In our experiments, the donor fluorophore mTurquoise2 was fused to ATGL and the acceptor sYFP2 was fused to HILPDA. The donor fluorophore mATGL_mTurquoise2 in the absence of acceptor displayed a mean fluorescence lifetime ($\tau$) of about 3000 ps (see Figure 5B, left: fluorescence intensity, middle: corresponding false colored fluorescence lifetime image, right: distribution of fluorescence lifetimes). The donor alone showed a narrow distribution. However, when mATGL_mTurquoise2 was expressed in the presence of mHILPDA-sYFP2, a strong reduction (35%, $p < 0.001$) in donor fluorescence lifetime was observed (Figure 5C and D). Interestingly, the average fluorescence lifetime distribution of the donor in the presence of acceptor showed a wider distribution (Figure 5E), suggesting different populations of interacting species.

**HILPDA inhibits ATGL with an IC$_{50}$ value in the low µM range**
To determine the half-maximal inhibitory concentration (IC$_{50}$) of HILPDA, lysates of ATGL288 expressing cells were incubated with increasing concentrations of purified full-length HILPDA. As shown in Figure 6A, HILPDA inhibited ATGL in a concentration-dependent manner and an IC$_{50}$ value of 1.9 µM. The C-terminal truncations HILPDA_1-28 (Figure 6B) and HILPDA_1-24 (Figure 6C) exhibited similar inhibition capacities (IC$_{50}$ values of 5.0 µM and 5.4 µM, respectively). The IC$_{50}$ value for hG0S2 inhibition of ATGL is 22 nM (Figure 6D) under the same experimental conditions and in agreement to previous reports (9). This approximately 100-fold difference suggests that G0S2 is a more potent inhibitor for ATGL than HILPDA.

**HILPDA is a highly selective inhibitor for ATGL**

Truncation studies of HILPDA indicate that the predominantly hydrophobic N-terminal region of HILPDA is essential for ATGL inhibition. Since hydrophobic regions are prone for non-specific protein-protein interactions, we analyzed the selectivity of HILPDA for the inhibition of different lipolytic enzymes. The screen included purified human MGL, purified bovine LPL and lysates of HSL expressing HEK-293 cells. HILPDA did not inhibit any of these lipases in enzyme activity assays (Figure 7). Hence, we conclude that HILPDA is a selective protein inhibitor of ATGL.

**Discussion**

Hypoxic conditions upregulate HILPDA expression and this response is often associated with increased TAG and LD accumulation. The data presented here clearly show that HILPDA can directly affect TAG catabolism by inhibition of ATGL. Decreased lipolytic activity can trigger metabolic effects in the organism, including decreased generation of lipotoxic intermediates, and decreased rates of oxygen-demanding fatty acid oxidation. The inhibitory effect of HILPDA on ATGL and TAG catabolism has been the subject of controversy. It is conceivable that the herein demonstrated IC$_{50}$ value of 2 µM of HILPDA for ATGL inhibition might explain the modest effects observed in literature reports. Similar IC$_{50}$ values have been reported for ATGL inhibition with long-chain acyl-Coenzyme A (28). We also identified the N-terminal region harboring the first 20 amino acids of HILPDA as the mediator for ATGL inhibition. ATGL, the target of HILPDA inhibition, consists of approximately 500 amino acids. Previous
studies have demonstrated that the N-terminal, patatin-domain containing part of the protein is essential and sufficient for TAG hydrolysis, interaction with the co-activator protein CGI-58/ABHD5, the murine-specific synthetic inhibitor Atglistatin, acyl-CoA, and the inhibitory protein G0S2 (9,10,28,35). This study reveals that HILPDA also targets the patatin-domain containing region of ATGL. Inhibition of the TAG-hydrolyzing activity of ATGL by HILPDA occurs independently of the presence of CGI-58 suggesting that binding of both proteins to ATGL is not mutually exclusive. Direct protein-protein interaction between HILPDA and ATGL is supported by immunoprecipitation experiments with full-length ATGL, TAG-hydrolysis studies, MST experiments with ATGL truncated at residue 288, and FRET-FLIM analysis. We also show that HILPDA is not a general inhibitor of lipases. While it inhibits ATGL activity, it has no effect on the other major lipolytic enzymes HSL and MGL or the vascular TAG lipase LPL.

The inhibitory function of HILPDA towards ATGL, along with its expression regulation, protein size, and amino acid composition, shares striking parallels with the previously characterized ATGL inhibitor G0S2 (Figure 1). G0S2 has evolved earlier during evolution as indicated by orthologs in sharks, latimera, gars and later developed vertebrates. In contrast, BLAST searches indicate that HILPDA is present only in two of the three groups of mammals, namely in eutherian mammals and marsupials (Monodelphis domestica). No HILPDA ortholog has been reported for egg-laying mammals (monotremes). Whether the gene coding for HILPDA evolved from a gene duplication event that was tolerated and probably provided a fitness advantage under certain environmental conditions, or if it appeared independently from G0S2 remains a matter of speculation. The human genes coding for HILPDA and G0S2 are located on different chromosomes (chromosome 7 and chromosome 1, respectively). The encoded proteins differ in size by 40 amino acids, which is, considering the size of HILPDA with only 63 residues, a quite substantial difference. Yet, we and other laboratories could show that the highly conserved N-terminal region of G0S2 includes the key residues involved in the interaction (3,9). In a similar manner, we could identify that the N-terminal region of HILPDA, which has the highest similarity to G0S2 is also sufficient to inhibit ATGL.
Previous reports identified HILPDA and G0S2 as LD associated proteins and the corresponding genes being targets of PPARs (13–16,18). Biochemical characterization, knockdown and overexpression studies have demonstrated uniformly, that G0S2 functions to attenuate ATGL-mediated lipolysis (9,36,37). Up to now, the effect of HILPDA in TAG catabolism had remained controversial. Depletion of HILPDA was reported to lower hepatic lipid accumulation, reduce fat pad weight and result in loss of lipid droplet formation in macrophages (13,14,16,18,38). Dijk et al. observed only modest changes in adipogenesis and lipolysis upon manipulations of HILPDA expression in adipocytes (15,18). Our in vitro experiments presented here demonstrate that in analogy to the function of G0S2, HILPDA is a direct inhibitor of ATGL. However, dose-response curves show a much higher IC50 value for HILPDA than for G0S2 (2 µM vs 22 nM for HILPDA and G0S2, respectively). This relatively low inhibitory capacity raises the question on the physiological role of HILPDA in the temporal and spatial regulation of ATGL-mediated lipolysis. Nevertheless, it should be kept in mind, that the data presented here are from in vitro experiments and thus might not directly reflect the physiological relevance of HILPDA-mediated ATGL inhibition, since the affinities and local concentrations might vary in the presence of artificial substrates and LDs. Since HILPDA is strongly induced under hypoxic conditions it is conceivable that it regulates lipolysis particularly under hypoxic or anaerobic conditions as often observed in rapidly growing malignant tumors. Similarly, HILPDA may regulate lipolysis during metabolic reprogramming when cells have restricted oxygen supply to enable higher ratios of adenosine triphosphate production per molecule of oxygen consumed and to reduce production of reactive oxygen species (39–41). In cultured myocytes, G0S2 serves as enhancer of ATP synthase thus enhancing cellular tolerance upon hypoxia (11). Whether HILPDA shares even more functions with G0S2 and also directly interacts with mitochondrial ATP synthase, or other protein interaction partners of G0S2 such as nucleolin (42), and/or Bcl-2 (43) is currently unknown and awaits further investigation.
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Table 1: Primers used for cloning of hHILPDA variants, HSL and ATGL

| Construct       | Forward Primer (5’-3’)                      | Reverse Primer (5’-3’)                      | Vector   |
|-----------------|--------------------------------------------|--------------------------------------------|----------|
| hHILPDA_1-63    | GTATTTTACAGGCGCCATGCG                       | AGTTGGTGTTGGTGTTGGTGCTTAAC                | pSumo    |
|                 | CATGAAGCATGTGTTGAC                         | AGCCTGCTACGTCGAC                         |          |
| hHILPDA_1-20    | CTCATCTTTCTAGAGGAGTGTG                       | AGTAGGGTCAGATGAGAGA                      | pSumo    |
|                 | GTAGTCC                                       | ACNAGATGAGAGT                            |          |
| hHILPDA_1-24    | TAGAGTAGTGATGATCCCTAGA                       | ACNAGATGAGAGT                            | pSumo    |
|                 | GGGCTTAC                                      | AGTGGTGGTGGTGGTGGTGGT                    | pSumo    |
| hHILPDA_1-28    | GTCCTAGAGGATTACTAGA                        | GCCCATGAGGACCCCATG                        | pSumo    |
| hHILPDA_20-63   | GTATTTCAGGGCGCCATGG                         | AGTTGGTGTTGGTGTTGGTGTGTACC               | pSumo    |
|                 | CTTGTTAGGATGGATG                            | ACAGTTTGCTGGAT                            |          |
| hHILPDA_25-63   | GAGTCCTAGAGGAGGCTTACTAGA                   | GCCCATGAGGACCCCATG                        | pSumo    |
| hHILPDA_1-63    | GCATTAGACGAGCAGCTGTAACAG                   | GCCATGGACGAGGATGAGAGA                    | pGFPC1   |
|                 | GAAAACCTGAATTTTCAAG                         | GCCATGGACGAGGATGAGAGA                    |          |
| mHSL            | GTCACTCGAGGGCCACCATGGG                       | GACTCCGCGGTTTCAGTTGGTGC                  | pECFP-N1 |
|                 | TTTCAGCAGATGAC                              | AGCAGGCG                               |          |
| mATGL_1-288     | AGGTGGCGGAGAAGAAGAAGACTG                     | GCAGTGATGATGATGATGATGATGATGATGATG          | pST50    |
|                 | ATTTTCAAGGCGACATG                           | GCCATCGGCTCGGAGCAGG                      |          |
| mHILPDA         | GCCCTGAGACCACATGAGAAGTAGCTTGCTACG            | ATGGTACCCGACTGCTACCTGGGGGAGG             | pEGFP-N2 |
|                 | GGGAGAGCAAG                                  | ATGGTACCCGACTGCTACCTGGGGGAGG             |          |
| mATGL           | GCCCTGAGACCACATGAGAAGTAGCTTGCTACG            | ATGGTACCCGACTGCTACCTGGGGGAGG             | pEGFP-N2 |
|                 | GGGAGAGCAAG                                  | ATGGTACCCGACTGCTACCTGGGGGAGG             |          |
| mCherry         | ATGGTACCATGGTGAGCAGGAGG                    | AGCGGCGCTCAGCTGACAGT                   | pEGFP-N2 |
|                 | GCCGAG                                | AGCGGCGCTCAGCTGACAGT                   |          |
| sYFP2           | ATGGTACCATGGTGAGCAAGGAGG                   | AGCGGCGCTCAGCTGACAGT                   | pEGFP-N2 |
|                 | GCCGAG                                | AGCGGCGCTCAGCTGACAGT                   |          |
| mTurquoise2     | ATGGTACCATGGTGAGCAAGGAGG                   | AGCGGCGCTCAGCTGACAGT                   | pEGFP-N2 |
|                 | GCCGAG                                | AGCGGCGCTCAGCTGACAGT                   |          |
FIGURES

Figure 1 Sequence and structural analysis of HILPDA. A. Sequence alignment of HILPDA and G0S2 from different species (md: Monodelphis domestica, m: Mus musculus, h: Homo sapiens, rt: Rhincodon typus). Residues highlighted in bold correspond to highly similar regions in the sequences of hHILPDA and hG0S2. Similarities and identities between HILPDA and G0S2 proteins are indicated directly below the sequences of the two proteins, those of both proteins are indicated in an extra line (bold). B, Phylogram of aligned sequences as in the previous panel. C, Secondary and tertiary structure prediction of HILPDA using Phyre2. Residues 2-23 aligned with the integrin alpha l transmembrane domain (pdb-code 2me3, unpublished) and are colored dark grey.
Figure 2 hHILPDA inhibits hATGL and mATGL under basal and CGI-58 stimulated conditions.

A, Coomassie stained SDS-PAGE gel of purified smt3-HILPDA fusion protein (22 kDa). B-E, Activity assays were performed in absence and presence (indicated by an asterisk ATGL*) of 1 µg purified CGI-58. 25 µg of ATGL lysates, 5 µg of purified HILPDA were used in all assays. 5 µg of purified hG0S2 or 5 µg of purified smt3 were used wherever it is specified. Values corresponding to 100% ATGL activity upon activation with CGI-58 (ATGL*) are mentioned in parenthesis below. TAG hydrolase activity of bacterial cell lysates expressing human (B, 343 nmol FA/h*mg protein) or mouse (C, 635 nmol FA/h*mg protein) ATGL288 in the absence or presence of purified CGI-58, HILPDA or smt3. TAG hydrolase activity of HEK-293 cell lysates expressing human (D, 634 nmol FA/h*mg protein) and mouse (E, 638 nmol FA/h*mg protein) ATGL in the absence and presence of purified CGI-58, HILPDA, or smt3. F, HEK-293 cell lysates co-expressing mATGL and HILPDA were tested for TGH activity levels under basal and CGI-58-stimulated conditions. HEK-293 cell lysates co-expressing mATGL (395 nmol FA/h*mg protein) and hG0S2 or empty vector served as controls. Statistical significance in comparison with ATGL* (white bar) was assigned according to the scheme: p < 0.05 (*), p < 0.01 (**), p < 0.001 (***). 

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Figure 3 The N-terminal region of HILPDA is essential for ATGL inhibition. A, Graphical representation of full-length and C- and N-terminal truncated versions of HILPDA. Variants capable of ATGL inhibition are colored in dark grey. B, Coomassie stained SDS PAGE gel of purified HILPDA variants expressed in E. coli. C, HILPDA variants were tested for their ability of ATGL inhibition. Activity assays were performed in the presence of CGI-58 as indicated by an asterisk (mATGL*). 5 µg of purified HILPDA protein was mixed with 25 µg of mATGL288 lysate and 1µg of purified mCGI-58. Purified smt3 was used as a negative control. 100% ATGL activity corresponds to 635 nmol FA/h*mg protein. Statistical significance in comparison with ATGL* (white bar) was assigned according to the scheme: p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), and, in comparison with HILPDA_FL (checked bar), according to the following scheme p < 0.05 (#), p < 0.01 (##), p < 0.001 (###) representing three independent experiments.
Figure 4 HILPDA directly interacts with N-terminal region of ATGL

A, Co-immunoprecipitation of mATGL_FL and HILPDA. HEK-293 cells overexpressing HILPDA and mATGL separately or in combination were lysed and incubated with antibodies directed against the Flag epitope for immunoprecipitation. Equal amounts of cell protein or immunoprecipitates were analyzed for the presence of antigens by immunoblotting. B, ATGL/HILPDA interaction demonstrated by microscale thermophoresis. 0.01 µmoles of purified sf-GFP_mATGL_288 was titrated against increasing amounts of unlabeled smt3_HILPDA_FL and fluorescence distribution changes over a temperature gradient were measured using the Monolith NT.115. A Coomassie stained SDS PAGE gel of the purified proteins used in the MST measurement is shown in the inset.
Figure 5 HILPDA and ATGL colocalize and physically interact intracellularly. Hepa 1-6 cells were transfected with mATGL-mTurquoise2 and mHILPDA-sYFP2 or mHILPDA-mCherry plasmids under lipid loaded conditions (1.2 mM fatty acid mix oleate and palmitate). Colocalization was observed by confocal microscopy and their interaction was assessed using FRET-FLIM. A, mATGL-mTurquoise2 and mHILPDA-mCherry partially colocalize in lipid loaded Hepa 1-6 cells. B, Fluorescence intensity image, fluorescence lifetime image and fluorescence lifetime distribution of mATGL-mTurquoise2 transfected cell and C, Fluorescence intensity image, fluorescence lifetime image and fluorescence lifetime distribution of mATGL-mTurquoise2 and mHILPDA-sYFP2 cotransfected cell. D, Histogram
of average Fluorescence Lifetime (τ) of mATGL_mTurquiose2 (N 20) in absence and presence of acceptor mHILPDA_sYFP2 (N 21). 

E. Average τ distribution histogram in absence (N 20) and presence of acceptor (N 21). Data are presented as mean ± SEM (p < 0.001 (***)).
Figure 6 HILPDA inhibits ATGL with an IC<sub>50</sub> value in the low µM range. Dose dependent inhibition of TAG hydrolase activity of mATGL288 by HILPDA variants and G0S2. 44 nM to 44 µM of purified HILPDA or 37 nM to 37 µM of purified G0S2 were mixed with 25 µg mATGL288 lysate in the presence of CGI-58 and TG hydrolase assays were performed. A, full-length HILPDA; B, HILPDA_1-28; C, HILPDA_1-24; D, full-length hG0S2.
Figure 7 HILPDA selectively inhibits ATGL. MGL activity was measured using rac-(1,3)-monooleylglycerol as substrate. TAG hydrolase activity of LPL and HSL was determined using triolein as substrate. All assays were performed in the absence or presence of purified full-length HILPDA. A, 40 ng purified human MGL was mixed with 1.3 µg HILPDA (100% activity corresponds to 0.5 mmol glycerol/h*mg protein). B, 33 ng purified bovine LPL was stimulated with 10 µl of FCS (fetal calf serum, and assayed in the absence or presence of 2.5 µg of HILPDA (100% activity corresponds to 1.1 mmol FA/h *mg protein). C, 25 µg of HEK-293 cell lysate expressing HSL-ECFP was incubated in the presence of 5 µg HILPDA (100% activity corresponds to 183 nmol FA/h *mg protein).