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Lipolysis is altered in MHC class I zinc-α_2-glycoprotein deficient mice

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Abstract **Non-conventional major histocompatibility complex class I molecules are involved in a variety of physiological functions, most at the periphery of the immune system per se. Zinc-α_2-glycoprotein (ZAG), the sole soluble member of this superfamily has been implicated in cachexia, a poorly understood yet life-threatening, severe wasting syndrome. To further ascertain the role of ZAG in lipid metabolism and perhaps the immune system, we inactivated both ZAG alleles by gene targeting in mice. Subjecting these ZAG deficient animals to standard or lipid rich food regimens led to increased body weight in comparison to identically treated wild-type mice. This phenotype appeared to correlate with a significant decrease in adipocytic lipolysis that could not be rescued by several pharmacological agents including β_3-adrenoreceptor agonists. Furthermore, in contrast to previously reported data, ZAG was found to be ubiquitously and constitutively expressed, with an especially high level in the mouse liver. No overt immunological phenotype was identified in these animals.**

Keywords: Major histocompatibility complex; MHC; HLA; Non-classical MHC

1. Introduction

Conventional major histocompatibility complex (MHC) class I molecules sample the intracellular antigenic repertoire, for presentation to the αβ T cell receptor of cytotoxic T lymphocytes, thereby initiating adaptive immunity [1]. They are defined by polymorphic, membrane-bound, MHC (chromosome 6 in man and 17 in mus)-encoded heavy chains which heterodimerize with the non-MHC encoded immunoglobulin (Ig)-like β_2-microglobulin (β_2m). Members of a distinct, heterogeneous lineage of MHC-I genes, including zinc-alpha-2-glycoprotein (ZAG), have been implicated in a variety of diverse, yet crucial physiological functions. These include anti-infectious/tumor immunity for MIC [2,3], iron homeostasis for HFE [4] and trans-epithelial transfer of IgG for FcRn [5].

ZAG is the sole bona fide soluble MHC-I protein (devoid of transmembrane and cytoplasmic sequences), consisting of a single polypeptide chain that is widely distributed in body fluids. ZAG's structure resembles that of an archetypical MHC-I molecule with a membrane distal α1–α2 ligand-binding superdomain that incidentally, does not appear to bind a peptide, but rather a small hydrophobic entity, most likely fatty acid [6,7]. Unlike most other class I genes (with the exception of human *MIC* glycoproteins) ZAG acts independently of β_2m [6].

ZAG was initially identified over 40 years ago [8], yet our knowledge of its biological function(s) remains rudimentary. The discovery that ZAG shares sequence identity with a long sought, lipid mobilizing factor (LMF), first implicated ZAG in lipid metabolism [9,10]. Subsequently, a variety of circumstantial evidence has supported the idea that ZAG play a role in cachexia [11], a severe life-threatening wasting syndrome defined by massive depletion of both adipose and skeletal muscle tissues. Cachexia occurs in a number of patients suffering from cancer, AIDS, and other chronic illnesses. Cachectic patients have indeed been found to have an elevated level of ZAG in both serum and urine that appears to parallel weight loss [9]. Both ZAG and LMF induce lipolysis directly by stimulation of adipocyte adenylyl cyclase, and in mice appear to produce a dose-dependent decrease in body weight [12].

However, most if not all the biological data on ZAG has stemmed from biochemical preparations of the molecule isolated from various biological fluids. The inherent heterogeneity of these preparations might explain the plethora of biological functions ascribed to this single molecule. In addition to cachexia, these include a role in prostate cancer [13,14], bladder cancer [15], frontotemporal dementia [16], obesity [17], regulation of melanin production by melanocytes [18], cell-cycle dependant inhibition of cell proliferation via, perhaps, down regulation of cdc2 cyclin dependant kinase [19], inhibition of the proliferation of hepatic stellate cells [20], a role in psoriatic skin via adverse reaction to interferon-γ treatment [21], action as a cell adhesion molecule [22], ribonuclease activity [23], etc. At the receptor-ligand level the situation is not clearer. What,

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Abbreviations: MHC, major histocompatibility complex; ZAG, zinc-α_2-glycoprotein; LMF, lipid mobilizing factor; IBMX, isobutylmethylxanthine; FK, forskolin; WAT, white adipose tissue; BAT, brown adipose tissue

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if any, receptor ZAG binds is not well established, a single report has suggested binding to β_3-adrenoreceptor [24]. There have also been conflicting data with regard to ZAG's expression pattern; both at transcriptional and/or protein level [25–27]. Finally, the possible involvement of ZAG in immunity remains an open question, especially as ZAG carries a putative hydrophobic ligand and in this sense is reminiscent of CD1, another non-conventional MHC-I molecule involved in presenting glycolipidic moieties to NKT cells [28].

Generation and analysis of ZAG deficient mice will therefore help answer a number of questions regarding the pathophysiology of ZAG.

2. Materials and methods

2.1. Gene targeting in embryonic stem cells and generation of ZAG deficient mice

The targeting construct was designed to independently delete the α1 and α2 domains of ZAG. To do this, four fragments were amplified using the oligonucleotide pairs shown (Supplementary Table S1) and E14.1 genomic DNA as a template. The fragments were sequentially cloned into pBluescript, and a *pgk-neo*^{r} gene was inserted into the *Xho*I site created in fragment 2. Restriction sites (*Eco*RI and *Bgl*II) were introduced to tag the truncated exons 1 and 2, which encode the α1 and α2 domains. The construct was digested with *Kpn*I and *Not*I and purified to remove plasmid sequence. Targeting was carried out as previously described [4]. Briefly, 20 μg of purified fragment was electroporated into 1×10^7 E14.1 ES cells and colonies picked after selection in G418. Two hundred and eighty two colonies were expanded in duplicate and one set screened by Southern blot analysis with an external probe (Fig. 1B). One correctly targeted clone (confirmed with multiple digests, 5′, 3′ and internal probes) was injected into C57BL/6 blastocysts. Chimeras were backcrossed once and further intercrossed to generate and to generate ZAG$^{-/-}$ mice (Fig. 1A). Mice were genotyped by PCR of tail DNA using the following oligos: ZAG (9292–9312): 5′ ACT CTG TGC CAG GCT CAG GTG 3′; ZAG (9747-9727) : 5′ ACC ACA GGT CAG TCT GAT TAC 3′; Pgk-neo 3′ (14,309) : 5′ GAG ATC AGC AGC CTC TGT TCC 3′; Endogenous = 456 bp, Neo insertion = 519 bp, Neo deletion = 550 bp. Total RNA from liver and kidney was isolated from wild-type and ZAG$^{-/-}$ mice, subjected to Northern blot analysis and probed with full-length ZAG and HPRT cDNAs (Fig. 1C). Mice used in this study – both knockouts and wild-types – were on a mixed C57BL/6 × 129P2/OlaHsd background.

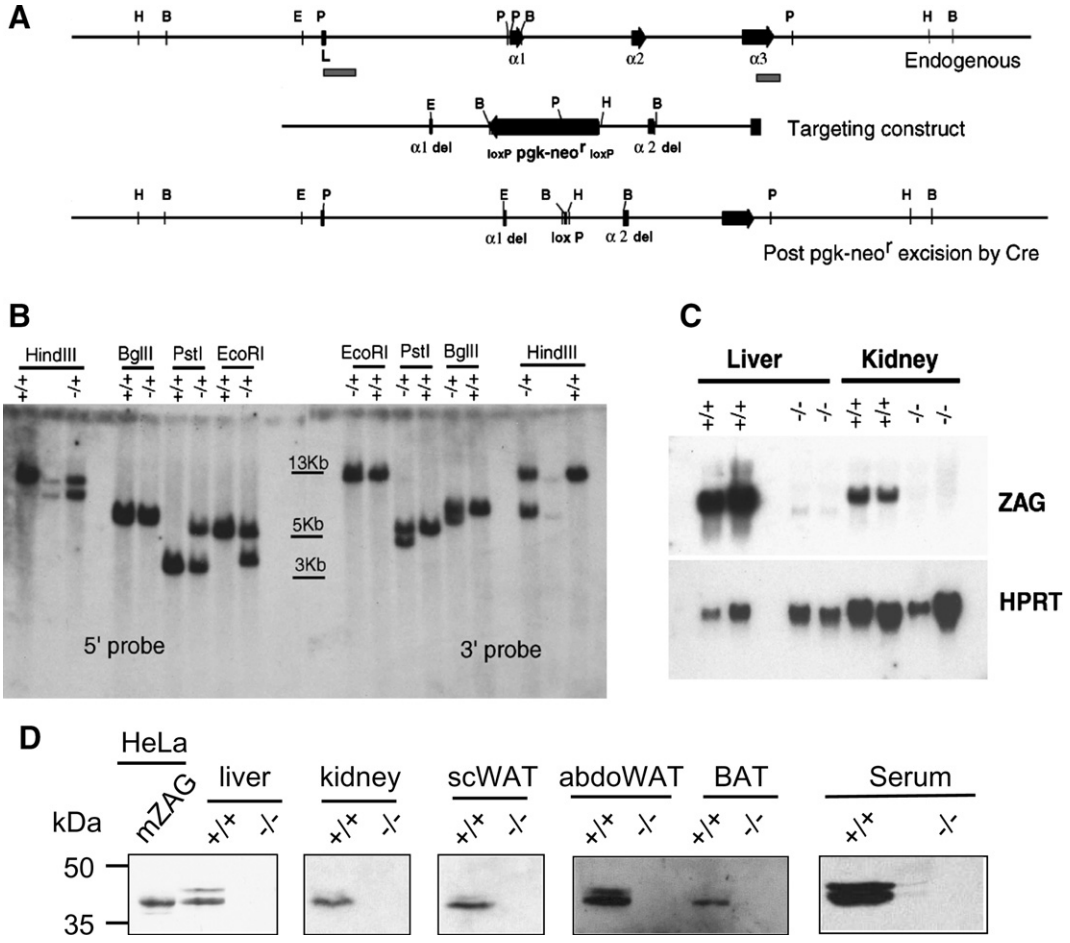

Fig. 1. Inactivation of ZAG by homologous recombination. (A) Scheme of endogenous *ZAG* (Top), targeting construct (Middle) and post *pgk-neo*^{r} excision by Cre (Bottom). (B) Southern blot of multiple digests of correctly targeted clone (+/−) and E14.1 control (+/+) using the 5′ (Left) or 3′ (Right) external probes. (C) Northern blot analysis of liver and kidney of homozygous mice ZAG$^{+/+}$ and ZAG $^{-/-}$. Two blots with the same samples loaded were probed either with ZAG cDNA (Top) or with HPRT cDNA (Bottom). (D) Western blot on lysates of HeLa cells transfected with mZAG as positive control and lysates from various organs from ZAG deficient and +/+ animals. *Abbreviation*: B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII: P: *Pst*I; *pgk-neo*^{r}: neomycine resistance gene driven by the phosphoglycerate kinase promoter; loxP: recombination sites for the Cre recombinase; ZAG$^{+/-}$: heterozygous ZAG mutant; ZAG$^{-/-}$: homozygous ZAG mutant; *HPRT*: hypoxanthine-phosphoribosyl transferase; WAT: white adipose tissue, BAT: brown adipose tissue; sc: subcutaneous; abdo: abdominal.

2.2. Plasmids and transfections

The full-length mZAG cDNA, including the endogenous secretory signal sequence was cloned from mouse liver. Total liver RNA was extracted using TRIZOL (Invitrogen, Cergy Pontoise, France). Upon reverse transcription, polymerase chain reaction was performed using "Expand Long template PCR System" following manufacturer's instructions (Roche Diagnostics, Meylan, France), together with the sense primer 5′ GCA AGA ATG GTG CCT GTC CT 3′ and reverse primer 5′ GGG TAA CTT ACT GAG GCT GA 3′. The unique band obtained at 930 bp was cloned into pCR2.1 using the TA cloning Kit (Invitrogen). The integrity of the sequence was verified by DNA sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, USA). The thus obtained mZAG cDNA was subsequently inserted into pcDNA3 expression vector. For transient expression, HeLa cells (0.2×10^6) were transfected using 6 μl of Jet PEI™ (Polyplus transfection, Illkirch, France) precomplexed with 3 μg of circular pcDNA mZAG. Forty-eight hours later, the cells were collected for further analysis. To generate stable ZAG expressing cell line, CHO-K1 cells (7×10^6) were transfected by electroporation with pcDNA mZAG. The medium was removed 48 h after transfection and replaced by fresh complete medium containing 0.5 mg/ml geneticin (Invitrogen). Individual geneticin resistant clones were picked out of the plate, expanded and subjected to limited dilutions in 96-well plates. The best mZAG expressing clones were further expanded.

2.3. Cell lysis and immunoblotting procedures

Proteins were isolated from HeLa and CHO-K1 cells transfected or not with pcDNA mZAG, and from mouse tissues, in the lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8, 0.5 mM EDTA, 1% NP-40, 10% glycerol) containing a cocktail of protease inhibitors (Complete EDTA-free, Roche Molecular Diagnostics, Meylan, France). Each tissue was destroyed using a Polytron. Cell and tissue lysates were left 15 minutes on ice and centrifuged at 4 °C 15 min at $13\,000 \times g$. The supernatant was kept at −80 °C for further analysis. Total protein concentration of each sample was determined using the Bradford Assay (Protein Assay, Biorad, Ivry sur Seine, France) and bovine serum albumin as a standard. Samples containing 40 μg of protein were mixed with Laemmli buffer and boiled for 5 min. Each sample was subjected to electrophoresis either on a 12% polyacrylamide gel (BioRad), or on a 12% ProSieve gel (Cambrex Bio Science, Emerainville, France), and semi-dry blotted onto Hybond-C Extra membrane (Amersham Pharmacia Biotech). Protein Rainbow molecular-mass standards (Amersham Pharmacia Biotech) were loaded simultaneously with the samples. Equal loading was confirmed by Ponceau S staining of the membranes (Sigma-Aldrich). ZAG was then revealed with the E-20 antibody (Santa Cruz Biotechnology, USA), using the ECL System (Amersham Pharmacia Biotech).

2.4. Tunicamycin treatment and deglycosylation

Sub-confluent cells (90-mm dish) were treated with tunicamycin (5 μg/ml) (Sigma-Aldrich) or with DMSO alone for 6 h at 37 °C in complete medium in humidified atmosphere containing 5% CO_2. The cells were lysed as described and the samples were separated on a 12% ProSieve gel (Cambrex Bio Science), and mZAG was revealed following immunoblotting. For deglycosylation, tissue or cell lysates (containing 100 μg of total protein in 50 μl of lysis buffer) were diluted in 50 μl of 2X incubation buffer (40 mM sodium phosphate pH 7.2, 0.2% SDS, 2% (v/v) β-mercaptoethanol, 1% NP40, protease inhibitors) and incubated overnight at 37 °C with shaking, alone or with 2 U *N*-glycosidase-F (Roche Molecular Diagnostics). Then, 20 μl of each samples were run on a 12% ProSieve gel and ZAG was revealed following immunoblotting with the E-20 antibody (Santa Cruz Antibodies).

2.5. Animal conditions and food intake

Animals were group-housed at 21 °C and had free access to food and water. Mice were weaned at the age of three weeks onto standard diet. At the age of five weeks, the groups were submitted to different diets with the following composition (as a percentage of total calories): Standard Food (SF: 320 kcal/100 g, protein 20%, fat 10% and carbohydrates 70%) ($n = 43$ wild-type and $n = 48$ knockout mice) or High Fat Diet (HFD: 556 kcal/100 g, protein 15%, fat 59% (lard), carbohydrates 26%) (Safe, Augy, France) ($n = 30$ wild-type and $n = 26$ knockout mice). The body weight was measured weekly from week 5 to 23. All experiments were performed on male animals. The quantity (weight) of food provided to each animal cage was monitored throughout the experiments.

2.6. Lipolytic assay

Epididymal adipose tissue was quickly dissected and adipocytes were isolated in Krebs–Ringer bicarbonate buffer containing collagenase (1 mg/ml). Fat cells were incubated at 37 °C in the presence of the different agents and shaken gently for 90 min, the glycerol released was determined and expressed as previously described [29].

2.7. Real-Time RT-PCR

Total RNA was isolated from different liquid nitrogen frozen tissues using either TRIZOL (Invitrogen), guanidium isothiocyanate followed by cesium chloride gradient, or RNeasy Lipid Tissue Midi Kit (Qiagen, Courtaboeuf, France). After reverse transcription of 1 μg of total RNA by ImProm-II™ Reverse Transcriptase (Promega), quantification of murine ZAG mRNA was performed with ABI PRISM 7000 (Applied Biosystems), using SYBR GREEN PCR Master Mix (Applied Biosystems). PRIMER EXPRESS software (Applied Biosystems) was used to design the following primers: ZAG: 5′ GGA CAC TAC AGG GTC TCA CAC CTT 3′ (forward), 5′ TGA AAT CCT CTC CGT CGT AGG C 3′ (reverse) and 18S: 5′ CGG CTA CCA CAT CCA AGG AA 3′ (forward), 5′ GCT GGA ATT ACC GCG GCT 3′ (reverse). The PCR parameters were as follow: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Amplification was performed in a final volume of 25 μl, and each sample contained 5 μl cDNA (equivalent to 12.5 ng of RNA), 300 nM of ZAG forward and reverse primers or 100 nM of 18S forward and reverse primers, and 12.5 μl of SYBR GREEN PCR Master Mix (Applied Biosystems). The size of ZAG and 18S amplicons were 107 bp and 186 bp, respectively. Each quantitative PCR reaction was performed in duplicate, and the mean value of Ct for each sample was used in data analysis. All samples were normalized to the 18S values and the results expressed as fold changes of Ct value relative to controls by using the $2^{-\Delta\Delta C_t}$ formula [30].

2.8. Biochemical and immunological parameters

All biochemical parameters were performed at the Clinique de la Souris (Illkirch, France) following standard procedures [31]. A preliminary phenotyping of lymphoid compartments was achieved using previously published methods [4].

2.9. Statistics

Statistical analysis was performed with StatView 5.0 software (SAS Institute, Cary, NC). The differences of body weight after SF or HFD feeding between $ZAG^{-/-}$ mice and normal mice was assessed with unpaired *t*-tests (Student's *t*-test). *P*-value < 0.05 was considered significant.

3. Results

ZAG was inactivated by homologous recombination in embryonic stem cells by mutating the second and third exons (Fig. 1A), leading to truncated α1 and α2 domains. Gene targeting of neomycin resistant clones was analyzed by Southern blotting (Fig. 1B). A heterozygote embryonic stem cell clone was used to generate mutant mice using standard procedures. $ZAG^{-/-}$ mutant mice were completely devoid of ZAG transcript and protein as, respectively, judged by Northern blot analysis of liver and kidney (Fig. 1C) and Western blotting of liver, kidney, fat depots and serum (Fig. 1D), heart, lung, stomach, intestine, spleen, brain and testis (data not shown). Lysates of HeLa cells transfected with murine ZAG (mZAG) cDNA were used as positive control (Fig. 1D).

Mice lacking ZAG were viable, fertile and displayed no gross abnormal phenotype. Throughout normal mouse

husbandry, it became apparent however, that occasionally, ZAG deficient mice displayed peculiar (quantitatively and qualitatively) fat repartition (cf. below). Given this observation and especially background knowledge on ZAG (see Section 1), and in order to conclusively gauge the intervention of ZAG in lipid metabolism, the following experiments were set up.

Body weight was monitored over a period of time ranging from the ages of 5 to over 23 weeks. Two large cohorts of ZAG deficient and wild-type littermate mice were constituted. One was fed with "standard food" (SF) whereas the other with a "High Fat Diet" alimentation (HFD). Although $ZAG^{-/-}$ gained significantly more weight than $ZAG^{+/+}$ control animals in both groups, this was more pronounced in the animals following HFD (Fig. 2). In addition to total body weight, individual organs were also regularly weighed, the livers from $ZAG^{-/-}$ mice were significantly heavier than those from wild-type animals (HFD diet) (data not shown). These cohorts of animals were assessed using a number of standard biochemical parameters. Among these the basal levels of glucose, insulin, triglycerides, free fatty acids and leptin were not significantly different in overnight fasted wild-type versus knock-out animals submitted to SF or HFD (data not shown). It should be noted that weight gain was independent of food intake as regular monitoring of such, did not show any significant difference between wild-type and knock-out animals.

Since ZAG has been shown to stimulate lipolysis in mice [10], we aimed to assess the state of lipolysis in the absence of ZAG. Lipolysis was induced by different agents e.g. forskolin (FK) that increases cAMP formation, isobutylmethylxanthine (IBMX) which augments also cAMP levels but through inhibition of phosphodiesterase activity, isoprenaline, a non selective β-adrenergic agonist and finally CL316243, a specific β_3-adrenergic agonist. As shown in Fig. 3, spontaneous basal lipolysis in epididymal adipocytes was not significantly

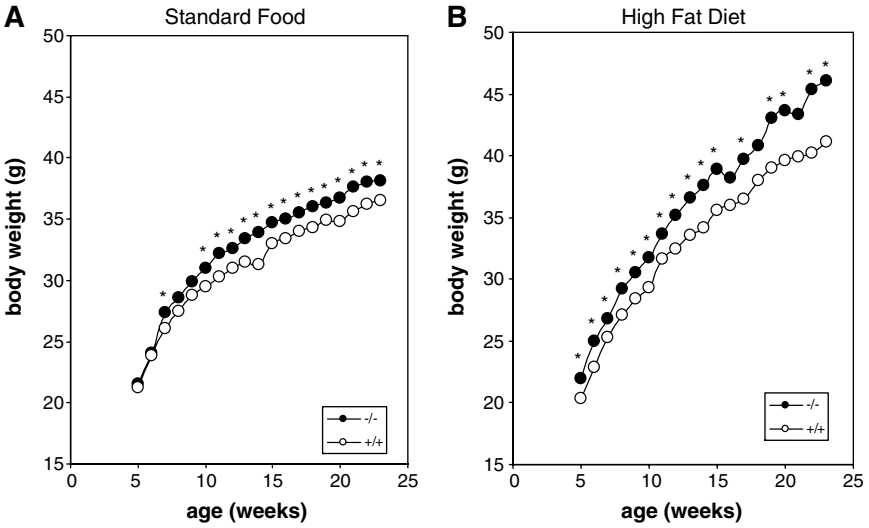

Fig. 2. Body weight curve of $ZAG^{+/+}$ (white circle) and ZAG $^{-/-}$ (black circle) Mice. (A) mice on standard diet, number of mice in each group, $n = 41–48$; *, $P < 0.05$. (B) Mice on high fat diet, number of mice in each group, $n = 26–32$; *, $P < 0.05$.

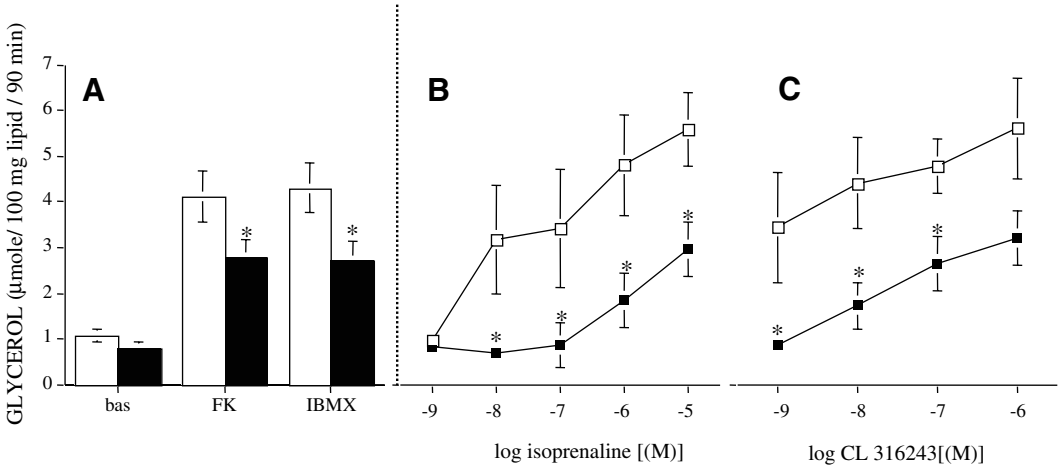

Fig. 3. Lipolytic activity of epididymal adipocytes isolated from wild type (white bar and square, $n = 6$) and $ZAG^{-/-}$ (black bar and square, $n = 7$) mice. (A) Effect of 10^{-5} M forskolin (FK) and 10^{-3} M isobutylmethylxanthine (IBMX) on basal lipolysis (bas). Results are means ± S.E.M. of six separate experiments. (B) Dose-dependent responses of isoprenaline and (C) CL316243. Results are means ± S.E.M. Comparison with results obtained in control mice was performed using Student *t*-test $^{*}P < 0.05$.

different and corresponded to 1.07 ± 0.16 and 0.74 ± 0.12 μmol / 100 mg lipid / 90 min glycerol release from adipocytes of $ZAG^{+/+}$ and $ZAG^{-/-}$ mice, respectively. Dose–response curves of both isoprenaline and CL316243 agents were significantly decreased in adipocytes of $ZAG^{-/-}$ mice, suggesting that this effect was not β_3-adrenergic specific. In addition, the lipolytic effects of FK (10^{-5} M) and IBMX (10^{-3} M) were significantly lower in adipocytes of $ZAG^{-/-}$ mice compared to controls. Thus, adipocytes of ZAG knockout mice presented a general decrease of lipolysis.

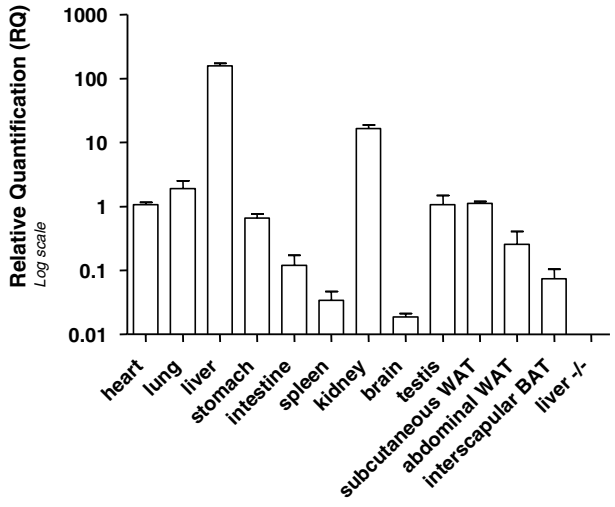

Fig. 4. ZAG gene expression in mouse tissues by real-time RT-PCR. mRNA levels were normalized to 18S RNA. Relative Quantification (RQ) of ZAG mRNA of heart, lung, liver, stomach, small intestine, spleen, kidney, brain, testis, subcutaneous WAT, abdominal WAT, interscapular WAT and BAT from wild-type mice and liver of $ZAG^{-/-}$ mice were given relative to heart. Results are means ± S.D. of 4–8 determinations on 2–4 different RNA extractions. They are represented in logarithmic scale.

The expression pattern of *ZAG* was assessed both at the level of mRNA and protein. Real-time quantitative RT-PCR (RT-qPCR) of ZAG transcript in various mouse tissues detected a strong signal in liver compared with weaker signals in all other tissues tested: heart, lung, stomach, intestine, spleen, kidney, brain, testis as well as various fat depots i.e. subcutaneous white adipose tissue (WAT), abdominal WAT and interscapular brown adipose tissue (BAT) (Fig. 4). However, each examined tissue did express ZAG, compared to the hepatic value in $ZAG^{-/-}$ mice which is zero. Western blot analyses were also performed in a number of $ZAG^{+/+}$ tissues. ZAG protein was observed in each tissue tested (Fig. 5A); the existence of multiple bands within the 38–43 kDa range was suggestive of glycosylation (there are three potential *N*-linked glycosylation sites in murine ZAG vs. 4 in man where actually three are glycosylated as assessed by X-ray crystallography). In order to be sure that these fluctuations are indeed associated with variations in the glycosylation state of ZAG [32], and that they are not non specific bands, deglycosylation and tunicamycin treatments were performed. A CHO cell line expressing mZAG was treated with tunicamycin. The cells were lysed and show ZAG totally deglycosylated at around 31 kDa (Fig. 5B). A CHO cell lysate expressing mZAG was treated with *N*-glycosidase F (Fig. 5B). In conclusion, deglycosylated ZAG and non-glycosylated ZAG migrate at the same level. Thus, each lysate of the tissues collected was treated with *N*-glycosidase F, and all of them showed the deglycosylated form of ZAG, as nothing is seen in $ZAG^{-/-}$ mice (Fig. 5C). Therefore, the characterization of wild-type control mice revealed that ZAG protein is ubiquitously and constitutively expressed, with an especially high level in the liver compared to other tissues tested.

4. Discussion

Given the initial (and even present) surprising structuring of ZAG/LMF within the MHC class I gene superfamily, one

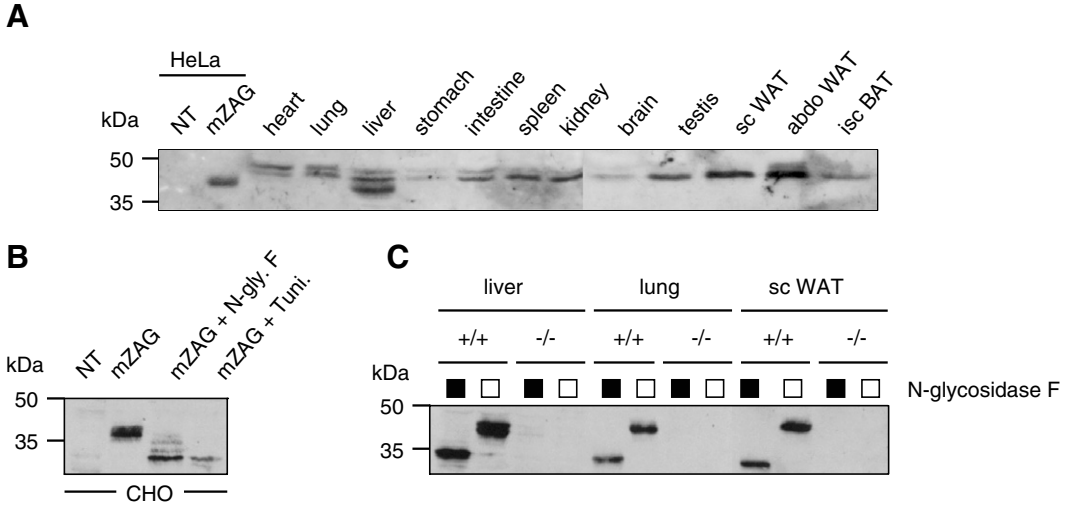

Fig. 5. ZAG protein in mouse tissues and in cell lines. (A) Western blot of ZAG in wild-type mice tissues and control HeLa cells or transfected with mZAG. (B) Effect of *N*-glycosidase F on ZAG in cell lysates and effect of tunicamycin in CHO cell lines expressing mZAG. (C) Effect of *N*-glycosidase F on tissue lysates obtained from ZAG +/+ and ZAG −/− mice. For Western blot, tissues were harvested in lysis buffer and destroyed using a Polytron. The polyclonal antibody E-20 directed against mZAG was used. CHO cells and CHO cells lysates were treated with tunicamycin or *N*-glycosidase F, respectively, as described in materials and methods. *N*-gly. F, *N*-glycosidase F; Tuni., tunicamycin.

would reasonably expect a role for ZAG in immunity. The most plausible scenario for the involvement of ZAG in the immune system would be through its capacity to bind and present, a lipidic entity, to T cells, not dissimilar from the interaction involving another non-conventional MHC class I molecule, CD1, which NKT cells [28]. Not withstanding this hypothesis, and comforted by several reports hinting at the capacity of ZAG to bind lipidic moieties [6,7,33], it is a general understanding that ZAG intervenes in the complex lipid metabolism pathway, although it will be of great help, if a simple genetic system was available to the examine the issue in live animals. Last, but not least, a myriad of papers have linked ZAG to a disconcerting range of biological activities, which collectively are difficult to reconcile with the action of a single molecule (cf. introduction), and therefore need to be sorted out.

In order to unequivocally probe the definite range of ZAG's pathophysiology, we ablated both ZAG alleles in the mouse genome through standard gene-targeting technology. Firstly, it was clearly shown that ZAG KO mice were overweight with respect to wild-type littermates inferring the opposite, i.e. ZAG overexpression should lead to weight loss and eventually cachexia, a result in line with the literature on the subject. Although, at gross anatomical level, this phenotype could not be ascribed to a single organ, given the episodic occurrence of abnormal fat repartition (as documented by Magnetic Resonance Imagery; data not shown) resembling lipodystrophic syndromes (which could not be systemically observed given most likely the mixed genetic background of studied cohorts) we decided to concentrate our efforts on the adipose tissue. The subsequent results on isolated adipocytes show a significant decrease of stimulated lipolysis in the absence of ZAG which could not be rescued using an array of agonists functioning upstream or downstream of adenylyl cyclase. These also included a β_3-adrenergic agonist: the CL316243 (Fig. 3). Although even lower concentrations of CL316243 could have been used to better characterize the dose–response curve, these data collectively demonstrate, that in contrast to previous reports, ZAG does not appear to signal via the adrenoreceptor pathway [24]. Moreover, the level of β_3-adrenoreceptor did not show any difference in $ZAG^{+/+}$ vs. $^{-/-}$ mice as evidenced by RT-qPCR (data not shown). These results are consistent with the fact that in human adipocytes, β_3-adrenoreceptor mRNA is expressed at a much lower level than in mouse [34]. Since ZAG leads to fat depletion in mouse and human, it is likely therefore that a common mechanism is used in both species. Finally, given the central role of Hormone-sensitive lipase (HSL) in lipolysis within adipocytes, we measured by RT-qPCR the level of HSL transcripts in adipocytes obtained from ZAG deficient and wild-type littermates. We could not observe any notable differences in the HSL levels nor could we do so for transcripts of other key molecules of lipid metabolism i.e. lipoprotein lipase (LPL) or fatty acid synthase (FAS) in the same setting (data not shown).

A bodywide screen helped establish the pattern of ZAG expression both at mRNA and protein levels, hence sorting out through the often limited and equally conflicting previously published results [25,27,35]. Our data clearly demonstrate that ZAG protein is ubiquitously and constitutively expressed in mouse, with an especially high level in the liver compared to other organs tested. The reason why there is relatively little ZAG mRNA present in most tissues, versus quite detectable quantities of protein might be one or several of the following generic situations: either ZAG mRNA is highly stable and/or the protein has a long half life, and/or ZAG is captured and internalized by low producing cells/tissues.

In conclusion, the work described here establishes a few facts about the enigmatic ZAG molecule. First, ZAG's expression pattern is much wider than previously thought and not limited to the adipose tissue or various epithelia. Second, in vivo ablation of ZAG in mouse is at the origin of significant weight gain which appears to correlate with a reduction of lipolysis in adipocytes. Finally, and although the immune system does not seem to be significantly altered in the absence of ZAG, subtle dysfunctions in minute, yet important, subsets of T cells could not be presently excluded. In fine, ZAG KO mice described here should prove to be a valuable tool to further characterize the role of ZAG in physiology, oncology, immunology and last but not least obesity and cachexia where a therapeutic use of the molecule may seem rational.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.12.047.

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