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Protein-Protein Interactions of human Zinc-alpha-2-Glycoprotein

MASTERARBEIT

zur Erlangung des akademischen Grades

Master of Science

Masterstudium Biochemie und Molekulare Biomedizin

eingereicht an der

Technischen Universität Graz

Betreuerin
Assoz. Prof. Mag. Dr. rer. nat. Robert Zimmermann

Institut für molekulare Biowissenschaften, BIOCHEMIE II
Karl-Franzens-Universität Graz

Graz, März 2020

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Danksagung

Ich möchte mich zu allererst bei meinem Betreuer Assoz. Prof. Mag. Dr. rer. nat. Robert Zimmermann bedanken, der diese Masterarbeit ermöglicht hat und mir immer mit Rat und Tat zur Seite stand. Auch möchte ich mich bei den Mitarbeiter_innen der AG Zimmermann und AG Lass bedanken, die stets zu Hilfe waren und für ein gutes Arbeitsklima gesorgt haben.

Mein größter Dank gilt natürlich meinen Eltern, Liane und Hans-Jürgen Neubauer, die mich in allen Lebenslagen liebevoll unterstützt und begleitet haben und ohne die das alles nicht möglich gewesen wäre.

Ich möchte mich auch bei all meinen Freunden bedanken, die für mich im und außerhalb des Studiums eine große Stütze waren.

Abstract

The human zinc-alpha-2-glycoprotein (ZAG) is a serum glycoprotein with a mass of approximately 40 kDa, that was first isolated from human serum by Burgi and Schmid. Since its first discovery in 1961, ZAG is the topic of many studies focusing on the physiological function of the protein. It is considered as a multifunctional protein contributing to many different processes in the human body. ZAG is secreted by a variety of tissues and is known to stimulate lipolysis in adipocytes. Elevated levels of ZAG in the urine of cancer patients also indicate a potential role as a biomarker for various types of cancer. However, the exact molecular mechanisms mediating ZAG effects are not completely understood.

In this master thesis, the protein-protein interactions of ZAG with human and murine adipocyte plasma membrane were investigated through chemical crosslinking. By the use of the biotinylated crosslinker Sulfo-SBED, recombinant human ZAG was cross-linked with human adipocytes and isolated murine adipocyte plasma membrane. The proteins were purified, taking advantage of the biotin-tag of the crosslinker. Using the label transfer method and immuno blotting, only weak interactions were observed with adipocyte membranes. ZAG primarily self-conjugated with itself indicating the formation of di- or oligomers.

Kurzfassung

Das humane Zink-alpha-2-Glycoprotein (ZAG) ist ein Serum-Glycoprotein mit einer Masse von ungefähr 40 kDa, das erstmals von Burgi und Schmid aus humanem Serum isoliert wurde. Seit seiner Entdeckung im Jahre 1961 ist ZAG Gegenstand vieler Studien, die sich mit seiner physiologischen Funktion befassen. ZAG ist als multifunktionelles Protein bekannt und ist an verschiedensten Prozessen im menschlichen Körper beteiligt. Es wird von zahlreichen Geweben sekretiert und zeigt eine stimulierende Wirkung auf die Lipolyse. Da im Urin von Krebspatienten erhöhte ZAG-Werte festgestellt werden, kommt das Protein womöglich als potentieller Biomarker für diverse Krebserkrankungen in Frage. Die molekularen Mechanismen über die ZAG Effekte vermittelt werden sind jedoch bis heute nicht vollständig erforscht.

In dieser Masterarbeit wurden Protein-Protein Interaktionen von ZAG mit humaner und muriner Adipozyten-Plasmamembran mit Hilfe von chemischem crosslinking untersucht. Durch den Einsatz des biotinylierten Crosslinkers Sulfo-SBED wurde rekombinantes humanes ZAG mit humanen Adipozyten und isolierter muriner Adipozyten-Plasmamembran kovalent verknüpft. Die verknüpften Proteine wurden mit Hilfe des Biotin-Tags des Crosslinkers aufgereinigt. Anhand der Label-Transfer Methode und Immunoblotting konnten nur schwache Interaktionen mit Adipozyten-Plasmamembran beobachtet werden. ZAG bildete primär Konjugate mit sich selbst, was auf die Formation von Di- oder Oligomeren hindeutet.

Contents

1 Introduction

1.1 Zinc-alpha-2-glycoprotein

The human zinc-alpha-2-glycoprotein (ZAG) is a serum glycoprotein with a molecular mass of approximately 40 kDa. It was first isolated by Burgi and Schmid in 1961 from human plasma and was named after its ability to form precipitates with zinc ions and its electrophoretic mobility in the alpha-2-globulin fraction [1]. ZAG was found to be secreted by a variety of tissues including breast, liver, prostate, gut [2] and adipose tissue [1]. Hence it is present in body fluids like serum, salvia, milk, sweat, urine and semen [1, 3, 4, 5]. ZAG was the topic of many studies investigating its structure and physiological role. However, the exact molecular function of the protein is still unknown.

1.1.1 Structure of Zinc-alpha-2-glycoprotein

The human zinc-alpha-2-glycoprotein consists of a 276 amino acid single polypeptide chain with four potential glycosylation sites [6]. As shown in figure 1.1, it is organized in three domains [7]. The α1 and α2 domains form a single eight-stranded antiparallel β-sheet with two α-helices on top, which is similar to the fold of major histocompatibility complex (MHC) class I proteins. The fold of the α3 domain resembles the conserved domains of immunoglobulin [8]. The crystal structure of human ZAG revealed a groove between the two α-helices of the α1 and α2 domains, which resembles the groove responsible for peptide binding of MHC class I molecules, but instead of a peptide, it seems to include an unidentified non-peptic ligand [8].

Due to the structural similarities with proteins of the MHC class I family, ZAG is considered an MHC-I-like protein and might be able to bind various ligands, antigens and proteins [7]. Unlike other MHC class I molecules, ZAG is a secreted protein, lacking the membrane spanning domain and it is not associated with β2-microglobulin, the light chain of MHC-I [8]. To visualize the structural similarities between ZAG and MHC-I molecules, the structure of MHC-I is shown schematically and as a ribbon diagram in figure 1.2. Both proteins share the overall structure of α1 and α2 domains, except for the transmembrane region and the β2-microglobulin of MHC-I.
A study published by Todorov et al. in 1998 showed that ZAG is also similar to a lipid-mobilizing factor (LMF) found in the urine of cancer patients with weight loss. Both proteins show homology in amino acid sequence and electrophoretic mobility, as well as the same chymotrypsin digestion pattern and immuno-reactivity [9]. Other studies confirmed, that ZAG and LMF were similar [10, 11, 12].

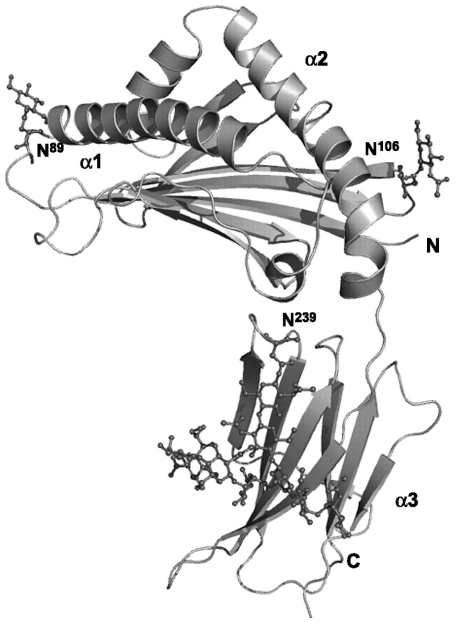

Figure 1.1: **Structure of ZAG.** Ribbon diagram of α1, α2, and α3 domains of zinc-α2-glycoprotein. Glycan chains represented in ball and stick model. The α1 and α2 domains form a single eight-stranded antiparallel β-sheet with two α-helices on top, which form a groove for binding of an unidentified non-peptic ligand. (source: Imtaiyaz et al. 2008 [7])

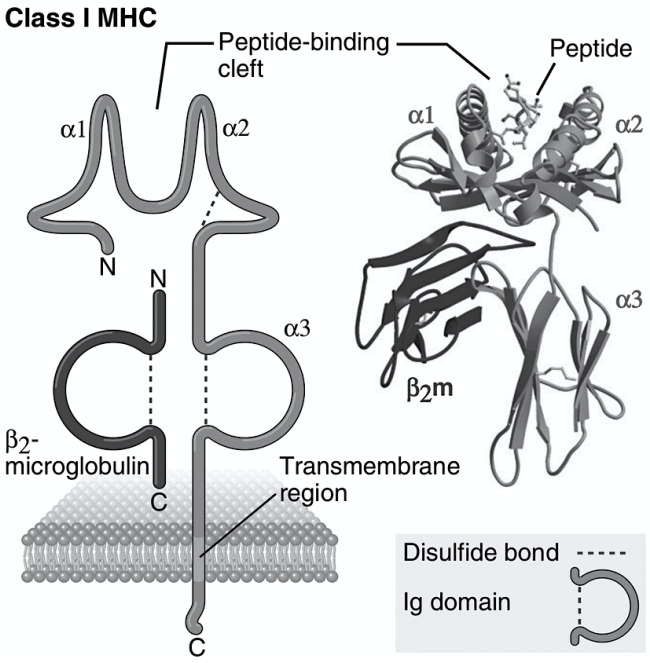

Figure 1.2: **Structure of MHC-I molecules.** Scheme and ribbon diagram of MHC class I. The peptide binding groove is formed by the α1 and α2 domains, the α3 domain contains a transmembrane unit. MHC-I is associated with its light chain, β2-microglobulin. (source: Abbas, Lichtman and Pillai, 2012 [13])

1.1.2 Physiological role

Although it is known since 1961, the various functions of ZAG are not completely understood yet. It is considered a novel adipokine and Mracek et al. found in 2010 that ZAG enhances adiponectin production of human adipocytes and is down-regulated with increased adiposity and circulating insulin. Therefore, it is suggested to be linked to obesity and obesity-related insulin resistance [2]. In a recent study, administration of ZAG was found to significantly increase insulin sensitivity in mice, whereas body weight, withe adipose tissue weight and intramuscular fat were significantly decreased compared to high fat diet mice fed [14]. A study published by Hirai et al. in 1998 already showed ZAG to stimulate lipolysis in vitro in isolated human and murine adipocytes. Mice treated with ZAG also showed a decrease in body fat mass without a change in body water or non-fat mass [10]. Furthermore, ZAG was found to stimulate adenylate cyclase in a GTP-dependant way in murine adipose plasma membranes [10]. A following study showed that

this stimulation is mediated by interaction with the β3-adrenergic receptor (β3-AR) [15].
As previously mentioned, ZAG was found to be synthesized in the prostate [16] and it is present in human ejaculates at concentrations fivefold higher than in blood plasma [5]. This indicates the suggested role of ZAG in fertilization. A study published by Ding et al. in 2007 showed, that it is related to sperm motility in human seminal plasma and may play a role during maturation of spermatozoa through fertilization [17].
Due to its structural similarities to MHC-I molecules, which are able to bind peptides and present it to cytotoxic T cells [18], some studies suggest ZAG to have a potential role in immune response [6, 8], however, this could not be proven yet. Another function of ZAG is its RNase activity that was published by Lei at al. in 1992. This study suggests, that the RNase activity of ZAG is comparable to onconase (a cytotoxic amphibian ribonuclease) and two orders of magnitude less than RNase A [19]. A following study on characterization of ZAG as a cell adhesion molecule found that proliferation of oral tumor cells is inhibited on a ZAG matrix and they suggested that the RNase activity might contribute to inhibition of proliferation [20]. He et al., in a study published in 2000, also showed a slow down in tumor cell proliferation in SiHa cells by addition of ZAG to the media as well as by transfection with ZAG cDNA. Both led to a reduction of cdc2, a key regulator of G2/M transition in the cell cycle [21], and therefore, reduction of proliferation, apparently without involving apoptosis or differentiation [22]. Since cdc2 is intensively upregulated in malign tumors, ZAG might hinder tumor progression by downregulation of cdc2 [22].

1.1.3 Pathophysiology

ZAG is associated with a number of diseases and as an adipocytokine, it might act as a link between adipose tissue and other organs. Wozny at al. investigated in 2019 the role of ZAG in chronic kidney disease and showed its contribution to progressive malnutrition in dialysis patients. Furthermore, ZAG was found in significantly increased levels in the serum of cancer patients, wich indicates a potential role as a biomarker for various types of cancer [23]. A study published

by Shibata and Miura in 1982 on nephritogenic glycoprotein in urine, which was thought to be only excreted as a consequence of glomerular basement membrane damage, revealed that ZAG appears to be a second source of nephritogenic glycoprotein in urine. This indicates that ZAG might act as a carrier protein of the nephritogenic glycoprotein [24]. ZAG also seems to be linked to breast cancer. A study published by Parris et al. in 2013 showed significantly lower levels of ZAG in invasive breast tissue compared to adjacent normal tissue. It is suggested, that four marker proteins, including ZAG, are critical for aggressive breast cancer phenotypes and may be used as therapeutic targets [25]. As described by Kong et al. 2010, downregulation of ZAG also occurs in pancreatic cancer and human recombinant ZAG was found to inhibit transforming growth factor-β-mediated tumor cell invasion and ERK2 phosphorylation in a ZAG-negative cell line. Therefore, it was identified as a novel tumor suppressor [26]. It is also suggested, that ZAG can be used as a biomarker for polycystic ovary syndrome [27] and prostate cancer [28, 29]. ZAG was also found in the urine and most prominently in invasive tumor cells of patients with superficial and invasive urinary bladder cancer, wich suggest a potential link between ZAG and invasive tumor development [30].
Another disease ZAG is potentially linked to, is psoriasis, an inflammatory disease characterized by hyperproliferation and aberrant differentiation of basal keratinocytes [31]. ZAG seems to be present in stratum corneum of normal epidermis and absent in psoriatic plaque [32].
As reported in a study published by Bing et al. in 2004, ZAG mRNA and protein levels are significantly increased in adipose tissue of mice with cancer cachexia, a catabolic state characterized by extensive weight loss. ZAG, with its lipid mobilizing activity, is suggested to contribute to the massive reduction of body fat and might influence the production of other adipokines [11]. Administration of ZAG to cachectic mice resulted in an increase of uncoupling proteins (UCP) in brown adipose tissue, skeletal muscle and liver [33]. UPCs are involved in the control of energy metabolism, leading to an increase of total energy expenditure and contributing to tissue waisting in cachexia [34]. Mice administered with ZAG also showed a dose-dependent decrease in body weight and the increased energy expenditure together with the lipolytic effect of ZAG seem to be responsible for the loss of adipose tissue, wich might indicate a potential role of ZAG in treatment

of obesity [12].

For a better understanding of the pathophysiology of ZAG, in 2007 Rolli et al. created a ZAG knock out (KO) mouse by gene targeting. The inactivation of both ZAG allels led to overweighted KO mice compared to wild-type littermates and isolated adipocytes showed a decrease of stimulated lipolysis in ZAG deficient mice [35]. ZAG KO mice do not seem to show significant alteration in the immune system, which challenges the structural based suggestion of a potential role of ZAG in the immun system [35]. A further study in ZAG KO mice showed resistance to cold-induced expression of browning markers in white adipose tissue and inhibition of body weight and adipose tissue weight loss in cold stressed mice, which contributes to the suggested therapeutic role of ZAG in treatment of obesity [36]. ZAG knock out mice also show an increase in expression of two major lipases, adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), in mammary glands, which is suggested to be a mechanism to compensate for the lack of ZAG [37].

1.2 Chemical cross-linking

Cross-linking is a method based on the formation of covalent bonds between proteins using cross-linking reagents as connectors. These reagents, called crosslinkers, are molecules consisting of two or more reactive groups and a spacer backbone. There are seven popular reactivity classes of reactive moieties: Carboxyl-to amine reactive, amine-reactive, sulfhydryl-reactive, aldehyde-reactive, hydroxyl-reactive, azide-reactive and photoreactive groups [38]. Photoreactive groups are non-reactiv until activation by ultraviolet or visible light. The reactive moieties of the crosslinker can form covalent bonds with specific functional groups like (primary) amines, carboxyls, sulfhydryls or carbonyls to link e.g. two interacting proteins.

There are two major types of cross-linking reagents, homobifunctional and heterobifunctional crosslinkers. Homobifunktional crosslinkers have identical reactive groups on both ends. They are used only in on-step reactions and form ran-

dom conjugates, which is ideal to capture a "snapshot" of all protein interactions. Heterobifunctional crosslinkers have different reactive groups and can be used in one-step reactions as well as for sequential conjugation. The later procedure is performed in two consecutive steps: in the first step the crosslinker binds to a protein by reaction of its most labile reactive group followed by removal of the excess crosslinker. In the next step, the labelled protein is covalently linked with an interacting protein by reaction of the second reactive group of the crosslinker. This procedure minimizes self-conjugation and random polymerization. Cross-linking reagents featuring a third moiety like a biotin label are referred to as trifunctional crosslinkers. For additional information see [38].

1.2.1 Sulfo-SBED

Sulfo-SBED (Sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido) hexano - amido] ethyl-1,3'-dithiopropionate) is a trifunctional cross-linking reagent consisting of an amine-reactive group, a photo-reactive group and a biotin label (figure 1.3, page 8). The amine-reactive sulfonated *N*-hydroxysuccinimide (Sulfo-NHS) active ester can form covalent bonds with primary amines at ph 7-9. The photo-reactive aryl azide has two modes of action, the short-lived nitrenes formed upon photolysis can interact non-specifically or undergo ring expansion and interact with nucleophiles, especially amines. Due to the fact that this crosslinker features a biotin label as well as a cleavable disulfide bond it is applicable for label transfer experiments and protein-protein interaction studies. For further information about this particular reagent see the Sulfo-SBED biotin label transfer reagent product manual by ThermoFisher Scientific [1].

[1] `https://www.thermofisher.com/order/catalog/product/33033#/33033`

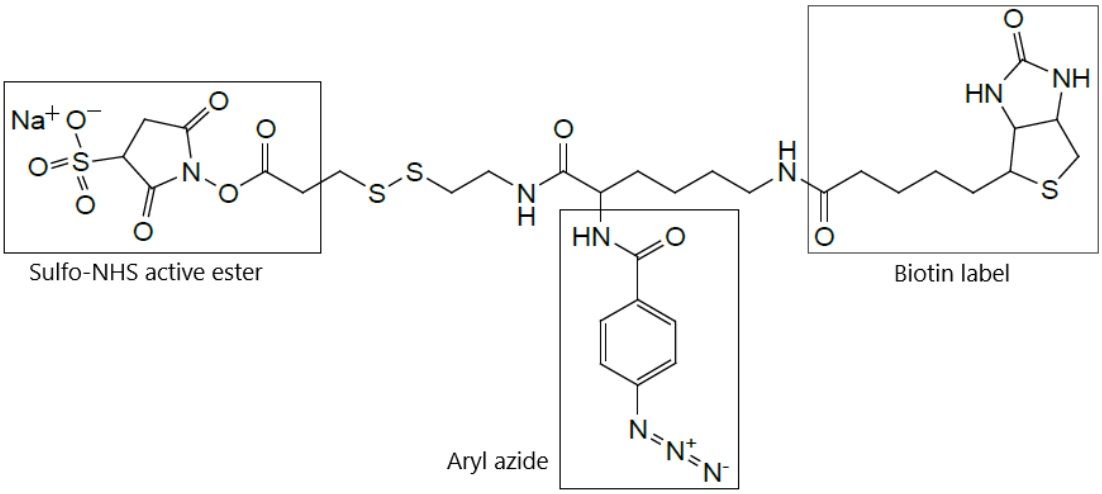

Figure 1.3: **Structure of Sulfo-SBED.** Trifunctional cross-linking reagent containing a sulfonated *N*-hydroxysuccinimide (Sulfo-NHS) active ester, which forms covalent bonds with primary amines at pH 7-9 and a photoreactive aryl azide reactive group, which is activated by UV light and form covalent bonds with nucleophiles. A biotin label and a cleavable disulfide bond enable label transfer experiments. (Source: Sulfo-SBED biotin label transfer reagent: User guide, Thermo Fisher Scientific)

1.2.2 The label transfer method

The label transfer method, as described by Thermo Fisher Scientific in their product manual, is used to capture interacting proteins by cross-linking with a biotin labelled cross-linking reagent, for instance, Sulfo-SBED. Therefore three reaction steps are performed which are depicted in figure 1.4) on page 9. The first step in the transfer is the biotinylation of a protein X by interaction with the sulfo-NHS ester group of the crosslinker. In the second step the biotinylated protein X is covalently linked to an interacting protein Y by the photoreactive aryl azide group, resulting in a biotinylated complex of protein X and protein Y. The last step, which is the name-giving step, is the reduction of the disulfide bond next to the sulfo-NHS ester group by the use of an reducing agent such as β-mercaptoethanol. The interacting proteins are separated, with the biotin label staying on protein Y. The label has been transferred from protein X in the beginning to protein Y in the end of the process. This label transfer can be visualized by western blot analysis using HRP-conjugated streptavidin or by the use of the photometric HABA (4'-hydroxyazobenzene-2-carboxylic acid) method. HABA has the ability to form

a complex with avidine which shows a specific absorption maximum at 500 nm. Addition of the biotinylated sample shows a change in absorbance.

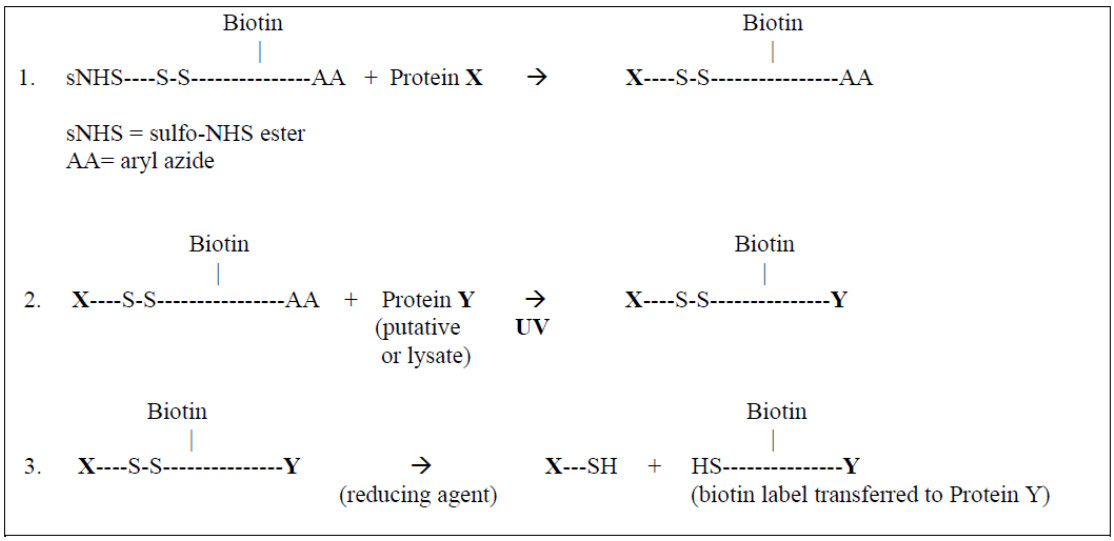

Figure 1.4: **Label transfer using Sulfo-SBED.** A protein X is biotinylated and linked with an interacting protein Y by the crosslinker Sulfo-SBED. Reduction of the disulfide bond of the crosslinker separates both proteins with the biotin label located on protein Y. (Source: Sulfo-SBED biotin label transfer reagent: User guide, Thermo Fisher Scientific)

2 Aim of the thesis

The aim of the thesis was the investigation of specific protein-protein interactions between human zinc-alpha-2-glycoprotein and adipocyte plasma membranes by chemical cross-linking and label transfer experiments.

Purified human zinc-alpha-2-glycoprotein was covalently linked with interacting proteins on the cell surface of human adipozytes as well as isolated plasma membrane form mouse adipose tissue. This was achieved by the use of the biotinylated cross-linking reagent Sulfo-SBED, which enables detection of interaction partners by the label transfer method.

3 Methods

3.1 Cloning

To investigate protein-protein interactions between human zinc-alpha-2-glycoprotein (ZAG) and adipocytes, the protein had to be overexpressed and purified. Therefore, it was necessary to clone the coding sequence of ZAG into a mammalian expression vector and attach specific tags for purification. Two constructs of human ZAG were designed, one with a C-terminal Glutathion-S-Transferase (GST) tag and one with a C-terminal histidine (His) tag.

3.1.1 Primer design

For amplification of the DNA sequence coding for human zinc-alpha-2-glycoprotein, specific primers had to be designed. The coding sequence of human ZAG was looked up in the NCBI Nucleotide Database and the first 18 to 20 nucleotides of the coding sequence were used for the complementary part of the forward primer. The last 12-15 nucleotides were used to generate a reversed compliment for the complementary part of the reversed primer. To determine optimal melting temperature and length of the primer pairs the web tool Tm Calculator [1] from the New England Biolab homepage was used. For correct integration into the two different mammalian expression vectors specific restriction sites had to be added to the primers. The composition of each primer pair and the specific restriction sites added are shown in table 3.1 on page 14. In order to facilitate subsequent purification, protein tags had to be added to the sequence. For the His-tagged construct this was achieved by cloning the coding sequence into the pcDNA4-myc-His-C mammalian expression vector, which adds a C-terminal histidine tag to the

[1] `http://tmcalculator.neb.com/#!/main`

Table 3.1: **Primer composition for amplification of human ZAG and Glutathione-S-transferase.** Primers hZAG fw and rw were used for coloning of C-terminally His-tagged, hZAG2 and GST primers for the GST-tagged construct.

name	restriction site	extension	complementary sequence
hZAG fw	BamHI	ACAGGATCCACC	ATGGTAAGAATGGTGCCTG
hZAG rv	NotI	ACAGCGGCCGC	GCTGGCCTCCCAG
hZAG2 fw	HindIII	ACAAAGCTTACC	ATGGTAAGAATGGTGCCT
hZAG2 rv	SalI/SpeI	ACAGTCGACACTAGTACC	GCTGGCCTCCCA
GST fw	SpeI	ACAACTAGTACC	TCCCCTATACTAGGTTATTG
GST rv	KpnI	ACAGGTACC	TCAGTCACGATGCGG

protein.

Since there was no mammalian expression vector available with a C-terminal GST-tag, the coding sequence for this tag had to be amplified separately. The coding sequence of human ZAG was amplified using primers which add three restriction sites to the sequence, HindIII on the N-term and SpeI followed by SalI on the C-term of ZAG. This enabled the cloning of ZAG and GST into the vector in two separate cloning steps. By the use of HindIII and SalI restriction sites, ZAG was cloned into the pECFP-N1 mammalian expression vector first. Then the plasmid was transformed in competent *E. coli* DH5α cells, purified and checked for correct integration of the insert. The GST-tag was then inserted into the ZAG-containing vector C-terminally of the ZAG insert, by the use of SpeI restriction site previously added to ZAG and KpnI on the vector. The arrangement of restriction sites for ZAG-GST cloning is illustrated in figure 3.1. For verification of correct primer design, in silico cloning was performed using the program Serial Cloner. The resulting ligation product was checked for correct orientation of the inserts and it was ensured that it is in frame with the protein tag.

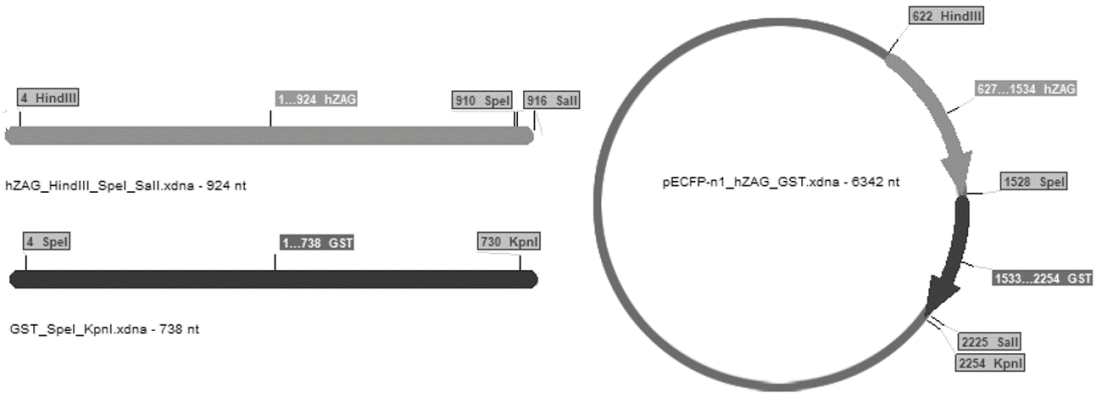

Figure 3.1: **Cloning of GST-tagged hZAG.**The map shows unique restriction sites addet to the inserts for construction of GST-tagged human ZAG. The image was created using Serial Cloner.

3.1.2 Polymerase chain reaction

The coding sequences of human zinc-alpha-2-glycoprotein and Glutathion-S-Transferase were amplified by polymerase chain reaction (PCR). As template for ZAG a plasmid vector containing human ZAG was provided by a colleague. To ensure it contained the right coding sequence and does not have any mutations the template was sent to Microsynth Austria GmbH (Vienna, Austria) for sequencing. The PCR of human ZAG was performed with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) in a Thermal Cycler with the following program. The initial denaturation was performed at 98°C for 30 seconds, followed by 34 cyles of denaturation at 98°C for 30 seconds, primer annealing at a temperature gradient from 55°C to 72°C for 30 seconds and primer extension at 72°C for one minute. The final extension was performed at 72°C for three minutes. For amplification of GST the bacterial expression vector pGEX-4T1, which features a GST tag was used as template. The PCR was performed under the same conditions as for ZAG, except the primer annealing was performed at 58°C for 30 seconds instead of a temperature gradient. For a list of components of the reaction mixtures of both PCR reactions see table 3.2 on page 16. To verify amplified DNA fragments the PCR products were applied for agarose gel electrophoresis with 1 % agarose gel in 1x tris-acetate-EDTA buffer (TAE, table 7.2, page 55) at 90 Volt.

Table 3.2: **PCR reaction mixture** used for am amplification of human ZAG.

Component	**25 μl reaction**	**final conc.**
5x Q5 reaction Buffer	5 μl	1x
1 mM dNTP mix	5 μl	200μM
1 μM fw primer	1,25 μl	0,5 μM
1 μM rv primer	1,25 μl	0,5 μM
0,1 μg/μl DNA template	1 μl	100 ng
Q5 HF Polymerase	0,5 μl	1 units
Water	11 μl	-

3.1.3 Purification of PCR products

For isolation of the PCR products from the agarose gel, the DNA bands were cut out and purified using the MicroElute Gel Extraction Kit (Omega Bio-tek Inc., Norcross, GA, USA). The purification was performed as per description in the product manual of the kit[2], the elution step was performed with 20 μl sterile deionized water.

3.1.4 Expression vectors

For generation of the His-tagged human ZAG construct the amplified DNA was cloned into the pcDNA4-myc-His-C mammalian expression vector. This vector adds a C-terminal histidine tag to the expressed protein and features ampicillin resistance for selection of transformed bacteria. Due to the lack of a mammalian expression vector with a C-terminal GST tag, the coding sequences of human ZAG and GST were cloned into the pECFP-N1 mammalian expression vector. The vector shows kanamycin resistance and adds a C-terminal fluorescent ECFP tag to the protein. Expression of the fluorescence tag was prevented by addition of a stop codon right after the GST coding sequence embedded into the extension of the reversed primer. More details on both vectors are shown in the vector maps in figure 3.2.

[2] `https://www.omegabiotek.com/product/e-z-n-a-microelute-gel-extraction-kit/`

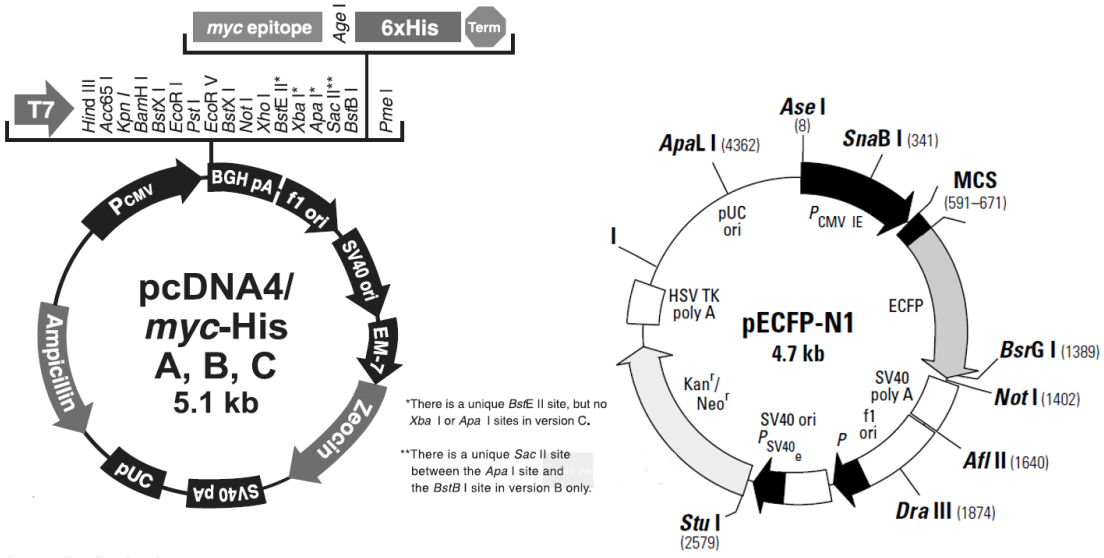

Figure 3.2: **Mammalian expression vectors** used for cloning of human ZAG.

3.1.5 Restriction digest

In preparation for the ligation of the vector and PCR products they had to be digested by specific restriction enzymes to ensure correct integration. For the His-tagged construct the insert and vector DNA was digested with the high-fidelity restriction enzymes BamHI-HF and NotI-HF (New England Biolabs). The components of the GST-tagged construct were digested using the high-fidelety restriction enzymes HindIII-HF and SalI-HF for cloning of ZAG into the pECFP-N1 vector and SpeI-HF and KpnI-HF (New England BioLabs) for cloning of GST into the ZAG containing vector. The reactions were performed at 37°C for 2,5 hours, followed by inactivation of the enzymes at 65°C for 20 minutes. All components of the reaction mixture and their amounts are listed in table 3.3 on page 18. To verify that all components were digested properly, they were applied for agarose gel electrophoresis with 1 % agarose gel in 1x TAE buffer at 90 Volt and isolated from the gel using the MicroElute Gel Extraction Kit (Omega Bio-tek Inc.).

Table 3.3: **Double digest reaction mixture**

Component	**50 μl reaction**
10x CutSmart Buffer	5 μl
Restriction enzyme 1	1 μl
Restriction enzyme 2	1 μl
Vector/insert DNA	1,5 μg
Water	fill up to 50 μl

3.1.6 Ligation

The Ligation of vector and insert DNA was performed at 16°C over night or at 25°C for 2,5 hours using T4 DNA ligase (New England BioLab) and correspondent buffer. After incubation, the enzyme was inactivated at 65°C for 10 minutes. To determine the correct vector to insert ratio the web tool NEBioCalculator Ligation Calculator [3] from the New England BioLab homepage was used.

3.1.7 Transformation of competent *E. coli*

After ligation, the recombinant plasmids were transformed into chemically competent *E. coli* DH5α cells to produce high amounts of plasmid for transfection of mammalian cell lines. For transformation of each construct 17 μl of competent cells were thawed on ice and mixed with 5 μl of the ligation mixture. After incubation on ice for 30 minutes the reaction tubes were heated in a water bath at 42°C for 30 seconds, followed by addition of 250 μl SOC-Medium (New England BioLab, preheated to 37°C) to the cell-DNA mixture. The mixture was incubated at 37°C and 180 rpm for one hour in a thermal cycler. 100 μl of the incubated cells were plated out on LB-ampicillin agar plates or LB-kanamycin agar plates for the His- and GST-tagged constructs respectively. A list of compounds of the different agar plates is shown in table 7.7 on page 57.
For Identification of transformants carrying the plasmid vector with a correctly integrated insert, 6 to 8 colonies were picked and cultivated at 37°C and 180 rpm over night in 3ml LB-medium (for composition see table 7.8, page 57). The plasmids were isolated on the next day using the innuPREP Plasmid Mini Kit 2.0

[3] `http://nebiocalculator.neb.com/#!/ligation`

(Analytik Jena AG, Jena, Germany). The plasmid preperation was performed according to protocol 1 of the user manual [4] provided by the supplier. Instead of using the provided elution buffer the elution was carried out using 30 μl sterile deionized water.

3.1.8 Verification of recombinant plasmid DNA

The isolated recombinant plasmids had to be checked for correct integration of the inserts and for mutations. Therefore, 1 μg of each plasmid DNA sample was digested by restriction enzymes which create a unique DNA fragment pattern on agarose gel if the insert is correctly integrated. The plasmid carrying His-tagged human ZAG was digested with the high-fidelity restriction enzyme SalI-HF (New England BioLabs). For the other construct this step had to be done twice. The vector containing only ZAG coding sequence was digested with SpeI-HF and NotI-HF, the final construct containing ZAG and GST was digested with NotI-HF (all from New England BioLabs). The reactions were performed at 37°C and 300 rpm for one hour in a thermal mixer (for the reaction mixture see table 3.4). After incubation the mixture was applied for agarose gel electrophoresis (1 % agarose gel in 1x TAE buffer, 90 Volt) and one positive sample of each construct was sent to Microsynth Austria GmbH (Vienna, Austria) for DNA sequencing. Therefore, 1,2 μg plasmid DNA was prepared according to the company's requirements and their appropriate standard primers for each plasmid were selected.

Table 3.4: **Reaction mixture for restriction digest** of recombinant vectors

Component	**30 μl reaction**
10x CutSmart Buffer	3 μl
Restriction enzyme	0,5 μl
Vector DNA	1 μg
Water	fill up to 30 μl

[4] `https://www.analytik-jena.de/produkte/kits-assays-reagenzien/kits-fuer-dnarna-extraktion/innuprep-plasmid-mini-kit-20/`

3.1.9 Plasmid preparation

The verified plasmids had to be transformed again in *E. coli* DH5α cells to produce enough plasmid DNA for overexpression of the proteins in in Expi293 cells. This was performed the same way described in 3.1.7 on page 18, but this time the over night culture was used to inoculate the main culture of 300 ml LB-Medium. For plasmid preparation the NucleoBond Xtra Midi Kit (Macherey-Nagel & Co. KG, Düren, Germany) was used. All steps were performed as per description in the product manual of the kit [5].

3.2 Cell culture

3.2.1 Transient overexpression in Expi293 cells

For transient overexpression of His- and GST- tagged human ZAG in Expi293F cells, the Gibco Expi293 Expression System (Thermo Fisher Scientific, Waltham, MA, USA) was used. The expression system is based on suspension-adapted human embryonic kidney cells and used for high-yield transient protein expression. For further information about this cell line and the composition of the medium see the user guide provided by the company [6]. The cells were cultivated in 25 ml suspension cultures with Gibco Expi293 Expression Medium (Thermo Fisher Scientific) in 125 ml Erlenmeyer flasks. The Expi293 cells were incubated at 37°C in a standard humidified 7 % CO_2 atmosphere on a shaking platform. Using the Expifectamine293 Transfection Kit (Thermo Fisher Scientific) the cells were transfected according to the user guide with 30 μg Plasmid DNA when they reached a density of $3 \cdot 10^6$ cells/ml. After 20 hours the two enhancers provided in the kit were added. The expressed protein is secreted into the medium and was collected after three days.

[5] https://www.mn-net.com/bioanalysis/kits/plasmid-dna/5122/nucleobond-xtra-midi-kit-for-transfection-grade-plasmid-dna?c=0

[6] https://www.thermofisher.com/de/de/home/life-science/protein-biology/protein-expression/mammalian-protein-expression/transient-mammalian-protein-expression/expi293-expression-system.html

3.2.2 Differentiation of human multipotent adipose derived stem cells (hMADS)

The aim of the thesis was to investigate protein-protein interactions between human ZAG and adipocytes. Therefore, one experiment was performed with differentiated human multipotent adipose derived stem cells (hMADS). The cells were cultivated and differentiated according to a protocol obtained from Christian Dani [39]. Cultivation of the cells was performed in 175 cm_2 cell culture flasks with growth medium containing basic fibroblast growth factor (FGF2) and incubation at 37°C in a standard humidified 7 % CO^2 atmosphere. The compounds of the growth medium are listed in table 7.9 on page 58. The cells had to be subcultured every three to four days before they have reached confluence. Therefore, the media was aspirated and the cells washed twice with 1x phosphate buffered saline (PBS, for composition see 7.1, page 55). The cell monolayer was covered with trypsin solution (Thermo Fisher Scientific) and incubated for two to three minutes under above mentioned conditions. Trypsinization was stopped by addition of 10 ml growth medium and suspension of the cells by pipetting. The cells were pelleted by centrifugation at room temperature and 250 x g for 5 minutes and resuspended in 2 ml growth medium for counting. Counting of cells was performed with the TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA) and $1,5 \cdot 10^4$ cells/ml were added to 20ml prewarmed growth medium and transfered into a 175 cm^2 cell culture flask. The medium was changed every other day.

For differentiation, the cells were seeded on 6-well plates at a density of 30.000 cells/cm^2 in growth medium containing FGF2. Two days after, the medium was changed to growth medium without FGF2. When the cells had reached confluence (after one or two days), the growth medium was changed for differentiation medium (table 7.10, page 58) containing insulin, rosiglitazone, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX). This medium was changed again after three days for growth medium without IBMX and dexamethasone. After a total of 19 to 22 days from the start of differentiation the adipocytes could be harvested.

3.3 Protein purification

3.3.1 Purification of His-tagged human ZAG

The His-tagged human ZAG protein was purified by metal affinity chromatography using TALON Metal Affinity Resin (Takara Bio Inc., Kusatsu, Shiga, Japan). To isolate the protein from the medium of transfected Expi293 cells, the cells were pelleted by centrifugation and the medium was collected. For 25 ml medium 400 μl Talon resin slurry was washed twice with 1x PBS and equilibrated with Talon resin equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7,0). The equilibrated slurry was added to the medium containing the protein and incubated over night at 4°C on a rotating wheel. On the next day the mixture was centrifuged at 4°C and 500 x g for 5 minutes and the pellet was washed three times with 1x PBS under the same conditions. The flow through of the first centrifugation step was collected and an aliquot of each washing step was taken for determination of protein concentration. After washing, the slurry was applied on a filtered centrifuge column and allowed to empty by gravity flow. The protein was eluted five times with 400 μl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM Imidazol pH 7,0) and the eluates were collected.

3.3.2 Purification of GST-tagged human ZAG

For purification of GST-tagged human ZAG, Glutathione (GSH) Sepharose 4B protein purification resin (GE Healthcare Life Sciences, Buckinghamshire, England) was used. The medium containing the protein was collected as described in 3.3.1 and 400 μl GSH-sepharose were washed three times with 1x PBS. The remaining steps were performed the same way as in above mentioned description, except for elution a different elution buffer (50 mM Tris, 10 mM GSH, pH 8) was used.

3.3.3 Colorimetric Protein Determination with Coomassie Blue - Bradford Assay

The concentration of the purified proteins was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories), which is based on the Bradford dye-binding method. The dye reagent contains coomassie blue G-250 and shows a spectroscopic shift in absorption maximum from 470 nm to 595 nm when reacting with proteins. This shift is proportional to the protein concentration of the measured sample. To quantify the amount of protein and subtract any background, a protein standard curve with bovine serum albumin (BSA) was measured. Therefore, BSA was diluted to concentrations of 1 mg/ml, 0,5 mg/ml, 0,25 mg/ml, 0,125 mg/ml, 0,063 mg/ml and 0 mg/ml in talon resin elution buffer and GST elution buffer for ZAG-His and ZAG-GST respectively. The assay was performed in duplicates in a 96-well plate, where 10 μl of the sample (or standard) were mixed with 200 μl of the dye reagent diluted 1:5 in sterile deionized water. The reaction was measured spectroscopically at 595 nm and the protein concentration was calculated on the basis of the standard curve. The proteins were dialysed against 1x PBS over night.

3.4 Protein interaction studies

To investigate protein-protein interactions between human ZAG and adipocyte plasma membranes, two different experiments were performed. In the first one, hMADS were incubated with Sulfo-SBED labelled human ZAG and in the second one, isolated plasma membrane from mouse adipose tissue was incubated with the labelled protein. Both experiments were followed by sodiumdodecylsulfate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE) under reducing and non-reducing conditions and western blot analysis to visualize a label transfer between labelled ZAG and interaction proteins.

3.4.1 Sulfo-SBED cross-linking of human ZAG and adipocytes

For interaction studies of human ZAG with human adipocyte plasma membranes, hMADS were seeded at high density and differentiated in 6-well plates. In preparation for cross-linking the medium was evacuated and the cells were washed with 1x PBS. To see differences between incubation of whole cells an cell lysates, in one approach cells were harvested and sonicated for 20 seconds at 15 % amplitude. The labelling of His- and GST-tagged ZAG and controls (GST and β-galactosidase) was performed with 3-fold molar excess of Sulfo-SBED in the dark at 4°C over night. The cells and lysates were incubated with the labelled proteins under the same conditions for one hour and exposed to UV light (365 nm) in a UVC 500 Ultraviolet Crosslinker (Hoefer, San Francisco, CA, USA) three times for 40 seconds with 5 minute breaks in between. The cells were cooled on ice during the whole procedure. After cross-linking the cells were scraped off in 1x PBS and sonicated twice for 20 seconds at 15 % amplitude followed by centrifugation at 4°C and 1000 x g for 5 minutes. The supernatant was centrifuged again at 4°C and 100.000 x g for one hour and the resulting pellet was resolved in 1x non-reducing SDS-sample buffer (for composition see table 7.3 on page 56).

3.4.2 Plasma membrane isolation

In the second experiment white adipose tissue of wild type mice was homogenized in 1x PBS / 1 mM ethylenediaminetetraacetic acid (EDTA) in a homogenizer and centrifuged at 4°C and 1000 x g for 10 minutes, the fat on top was discarded. An aliquot was taken to later demonstrate accumulation of the plasma membrane and the pellet and supernatant were centrifuged at 4°C and 35.000 rpm for one hour in an ultra centrifuge. After this step the supernatant was discarded and the pellet was resuspended in 2 ml of 20 mM phosphate buffer /1 mM EDTA at pH 7,4 and another aliquot was taken. The suspension was centrifuged again at 4°C and 35.000 rpm for one hour on top of a 1,12 M sucrose solution. The plasma membrane fraction which stays on top of the sucrose solution was collected and dialysed against 1x PBS overnight.

To demonstrate the accumulation of the plasma membrane during isolation, the concentration of free cholesterol was measured after every step. Therefore, the free cholesterol determination reagent from Greiner Laboratories GmbH (Flacht, Germany) was used. To create a standard curve, the Cholesterol Standard FS (DiaSys Diagnostic Systems GmbH, Holzheim, German) was diluted to concentrations between 0 and 100 mg/dl. To measure free cholesterol, 10 μl of each standard and plasma membrane aliquot was mixed with 100 μl of the reagent in a 96-well plate and incubated at 37°C for 5 minutes. After incubation the extinction was measured at 560 nm and the concentration was calculated on the basis of the standard curve. The protein concentration in the aliquots was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) and the amount of free cholesterol per mg protein was calculated.

3.4.3 Sulfo-SBED cross-linking of human ZAG and murine adipocyte plasma membrane

For this experiment only His-tagged human ZAG and the controls BSA, Pigment epithelium-derived factor (PEDF) and Retinol binding protein 4 (RBP4) were labelled with a 3-fold molar excess of Sulfo-SBED as described in 3.4.1 on page 24. The proteins were applied on size exclusion chromatography columns (Bio-Rad Laboratories) before labelling for additional purification and once again after labelling to remove excess crosslinker. The isolated plasma membrane was incubated with the labelled proteins for 30 min at 4°C in the dark and transferred to glass vials for UV exposition. This was performed at 365 nm in a UVC 500 Ultraviolet Crosslinker (Hoefer) three times for 5 minutes with 5 minute breaks in between and cooling on ice. After cross-linking the samples were centrifuged at 4°C and 100.000 x g for one hour and the pellet was resuspended in 1x non-reducing SDS-sample buffer.

In a second approach the centrifugation step after cross-linking was replaced with purification of the biotinylated proteins using streptavidin agarose (Thermo Fisher Scientific). To increase sensitivity the cross-linked proteins were purified by taking advantage of the biotin-tag and it's high affinity to streptavidin. For each cross-

linked sample 100 μl 50 % streptavidin agarose slurry was washed four times with 1x PBS / 1 % NP-40 and added to the samples. After incubation at 4°C over night on a rotating wheel, the slurry was applied on a filtered centrifuge column and allowed to empty by gravity flow. The samples were was washed five times with 1x PBS / 1 % NP-40 and eluted twice with 50 μl 1x non-reducing SDS-sample buffer and heating-up to 95°C.

To investigate if the proteins were self-conjugated in the cross-linking process, an aliquot of each sample was purified again using streptavidin agarose. This time, the columns were washed four times with 100 mM β-mercaptoethanol / 0,1 % SDS to reduce the disulfide bond and remove the initially labelled proteins ZAG, PEDF and RBP4. The interaction partners stay bound to the slurry by the biotin-tag and were eluted with 50 μl 1x non-reducing SDS sample buffer and heating-up to 95°C.

3.4.4 SDS-Polyacrylamide-Gel-Electrophoresis

In order to analyse the cross-linked proteins by western blot analysis and see the label transfer, they had to be separated by their size through Sodiumdodecylsulfate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE). To visualise the transfer of the biotin-tag from the labelled proteins onto their interaction partners, SDS-PAGE was performed twice, under non-reducing and reducing conditions. For non reducing conditions 20 μl of the samples already dissolved in 1x non-reducing SDS-sample buffer were heated to 95°C for 10 minutes and applied on a 10 % polyacrylamide gel. For reducing conditions 20 μl of the same samples were mixed with 100 mM β-mercaptoethanol, heated to 95°C for 10 minutes and applied on a 10 % polyacrylamide gel (for composition of separation and stacking gel see table 7.4 on page 56). The electrophoresis for each gel was performed at 25 mA for one hour in SDS-PAGE running Buffer (200mM Tris, 1,6 M Glycin, 0.83 % SDS).

3.4.5 Western Blot analysis

After electrophoresis the separated proteins were transferred onto a nitrocellulose membrane by western blotting. This was performed at 200 mA for 70 minutes in CAPS buffer (10 mM N-cyclohexyl-3-aminopropanesulfonic acid, 10 % Methanol, pH 11). The membranes were submerged in 10 % skimmed dry milk in 1x TST buffer (1,5 M NaCl, 500 mM Tris, 1 % Tween 20, pH 7,4) at 4°C over night or at room temperature for one hour to block unspecific epitopes. After blocking, the membranes were washed three times with 1x TST for 10 minutes. To detect ZAG, the membranes were incubated with the primary rabbit anti-zinc-alpha-2-glycoprotein antibody ab117275 (Abcam, Cambridge, United Kingdom; 1:2000 in 5 % skimmed dry milk) at room temperature for one hour. After washing again three times under the same conditions, the membranes were incubated with the secondary goat anti-rabbit-HRP antibody (Thermo Fisher Scientific, 1:10000 in 2 % skimmed dry milk) at room temperature for one hour. The blots were washed again three times with 1xTST buffer and detected through ELC reaktion with the ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.). To detect the biotin-tag on the same blots, the used antibodies had to be removed by submerging the membranes in 20 ml stripping buffer (62,5 mM Tris, 2 % SDS, pH 6,7) with 140 μl β-mercaptoethanol. The stripping was performed at 55°C for 20 minutes in a shaking water bath and followed by 4 to 5 washing steps with 1x TST for 10 minutes. The stripped membranes were blocked with 5 % BSA in 1x TST at 4°C over night or at room temperature for one hour, followed by another three washing steps with 1x TST. For detection of the biotin-tag, the membranes were incubated with HRP-conjugated streptavidin (Thermo Fisher Scientific, 1:5000 in 5 % BSA) at room temperature for 40 minutes. After another three washing steps in 1x TST the biotinylated proteins were detected through ELC reaktion with the ChemiDoc Imaging System (Bio-Rad Laboratories, Inc.). To visualize equal protein loading the membranes were stained with Coomassie brilliant blue after detection and destained with destaining solution. For composition of both solutions see tables 7.5 and 7.6 on page 56.

4 Results

4.1 Cloning

In order to overexpress human ZAG with a His or GST protein tag in Expi293 cells, the coding sequence for the protein had to be cloned into two different mammalian expression vectors, pcDNA4-myc-His-C and pECFP-N1. After transformation of competent *E. coli* DH5α cells and isolation of the plasmids, the plasmid DNA was checked for correct integration of the inserts and mutations by restriction digest and sequencing.

4.1.1 Cloning of His-tagged human ZAG

Polymerase chain reaction

For the amplification of human ZAG coding sequence for the His-tagged construct, a plasmid vector containing ZAG was provided by a colleague. To ensure there are no mutations in the template and confirm that it contains the right sequence, 1,2 μg of the DNA were sent to Microsynth Austria GmbH for sequencing. The sequencing report did not show any mutations. Therefore, the vector could be used as template for PCR. In order to find ideal conditions for amplification of ZAG, the PCR was performed at a temperature gradient from 55°C to 72°C, as described in 3.1.2 of the methods section. The used primers add restriction sites for BamHI and NotI to the sequence, which should yield an amplification product with a size of 917 bp. As shown in figure 4.1, ZAG could be amplified at all different temperatures.

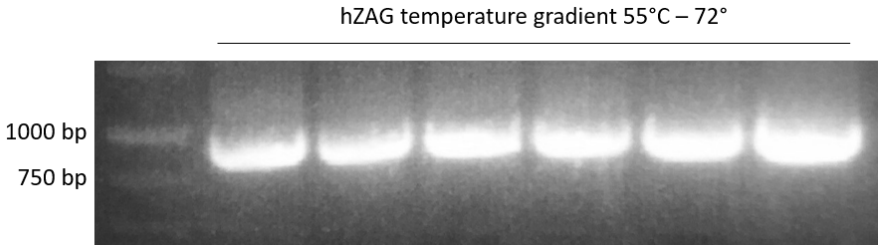

Figure 4.1: **PCR of human ZAG.** The PCR was performed with Q5 DNA polymerase. As template for ZAG a plasmid vector containing human ZAG was used, the used primers add restriction sites for BamHI and NotI to the sequence, resulting in a 917 bp PCR product.

Restriction digest

The amplified PCR products were cut out of the agarose gel and purified using the MicroElute Gel Extraction Kit (Omega Bio-tek Inc.). Insert and vector DNA were digested with the high-fidelity restriction enzymes BamHI-HF and NotI-HF as described in 3.1.5 . The restriction digest was verified by agarose gel electrophoresis, which should show the linearised vector with a size of 5071 bp and a 917 bp fragment for the insert DNA. As depicted in figure 4.2, DNA bands of the correct sizes are visible for both, vector and insert. The bands were cut out and purified again using the MicroElute Gel Extraction Kit (Omega Bio-tek Inc.) in preparation for the ligation reaction.

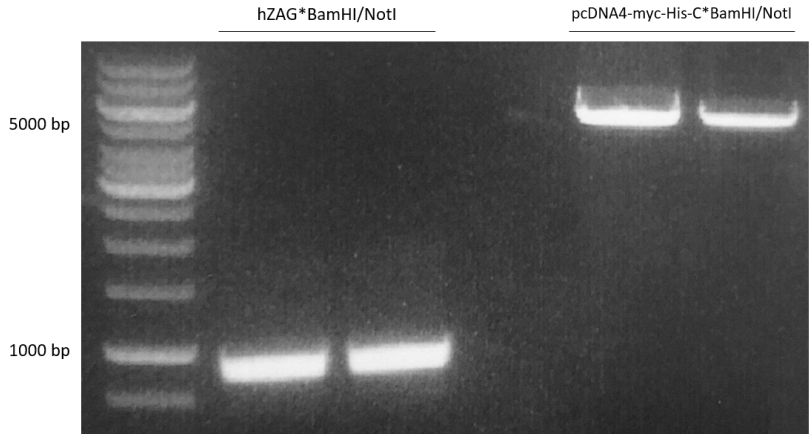

Figure 4.2: **Restriction digest of hZAG and pcDNA4-myc-His-C.** The used restriction enzymes BamHI-HF and NotI-HF yield DNA fragments of the size of 5071 bp and 917 bp for the vector and insert DNA respectively.

Ligation and transformation

The ligation of vector and insert DNA and the transformation of competent *E. coli* DH5α cells was performed as described in the methods chapter REF. After transformation, a few colonies were tested for correct integration of the ZAG insert by restriction digest with SalI-HF. If the insert is correctly integrated into the vector, the restriction digest yields two DNA fragments the size of 3703 bp and 2188 bp. As shown in figure 4.3, all tested colonies contained the correctly integrated insert. To ensure that the recombinant plasmid does not have any mutations, it was sent for sequencing to Microsynth Austria GmbH. The sequencing report did not show any mutations in the ZAG coding sequence (not shown).

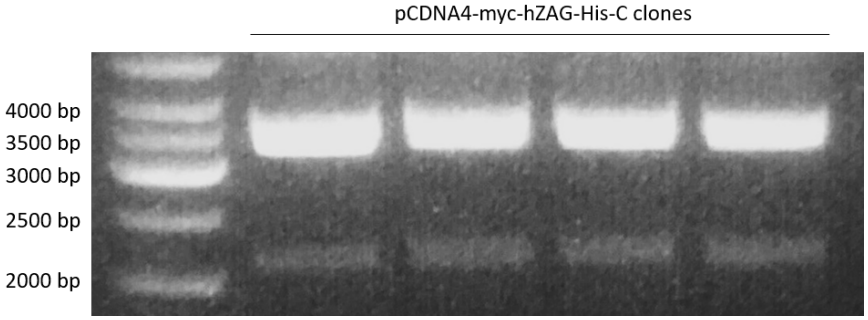

Figure 4.3: **Restriction digest of pcDNA4-myc-hZAG-His-C.** The used restriction enzyme SalI-HF yield DNA fragments of the size of 3703 bp and 2188 bp if the insert is correctly integrated into the vector.

4.1.2 Cloning of GST-tagged human ZAG

Polymerase chain reaction

For cloning of the GST-tagged ZAG construct, ZAG had to be cloned into the pECFP-N1 expression vector together with the GST-tag, since there was no mammalian expression vector available with an in-built C-terminal GST-tag. The first attempt to achieve this was to digest and ligate the amplified ZAG and GST sequences before ligation into the plasmid vector. As template for GST, the bacterial expression vector pGEX-4T1, which features a GST-tag was used. The used primers were designed to add restriction sites for HindIII and SacI to ZAG and for

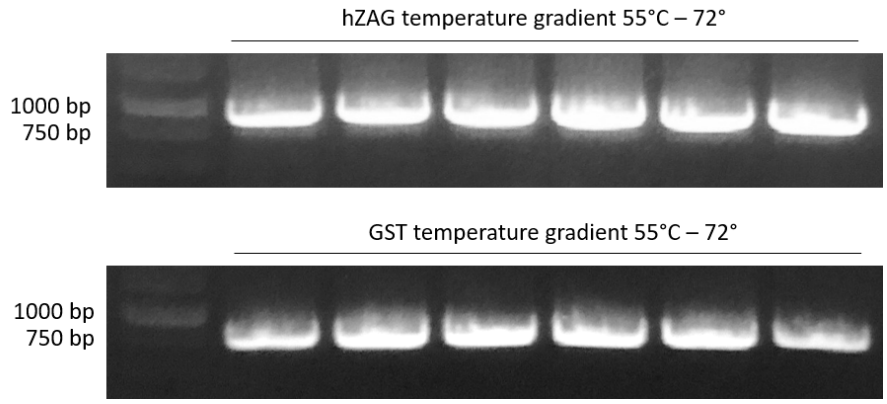

Figure 4.4: **PCR of human ZAG and GST.** The PCR was performed with Q5 DNA polymerase. As template for ZAG a plasmid vector containing human ZAG was used, GST was amplified from the pGEX-4T1 plasmid vector. The used primers add restriction sites for HindIII, SpeI and SalI to ZAG (924 bp) and SpeI and KpnI to GST (738 bp).

SacI and KpnI to GST. Both fragments could be amplified by PCR, but ligation of the products and cloning into the plasmid vector did not work. Hence, a different method to clone both inserts into the vector had to be found. The second approach was to clone ZAG into the vector first, then digest the ZAG containing vector again for ligation with GST. The PCR was performed under the same conditions with the same template DNA, but this time primers were used to add three restriction sites to ZAG. A N-terminal one for HindIII and two consecutive C-terminal sites for SpeI and SalI. HindIII and SalI were used for ligation into the vector and SpeI for ligation with GST. The primers used for GST amplification were designed to add restriction sites for SpeI and KpnI for ligation into the ZAG-containing vector. Amplification with these primers yield DNA fragments of 924 bp and 738 bp for ZAG and GST respectively. As shown in figure 4.4, ZAG and GST could be amplified at different temperatures from 55°C to 72°C.

Restriction digest of pECFP-N1 and ZAG

The amplified inserts were cut out of the gel and purified the same way as for the His-tagged construct. For the first step, which was the cloning of ZAG into the pECFP-N1 vector, insert and vector DNA was cut with the high-fidelity restriction enzymes HindIII-HF and SalI-HF. In figure 4.5, the linearised vector and the insert DNA can be seen as bands the size of 4733 bp and 924 bp, respectively.

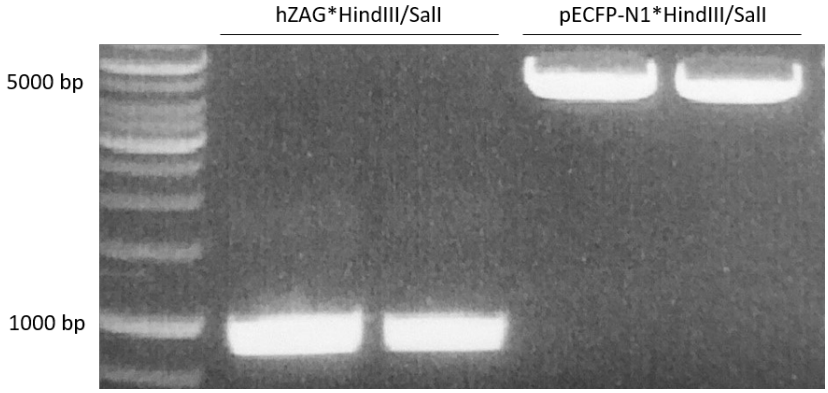

Figure 4.5: **Restriction digest of hZAG and pECFP-N1.** Vector and insert DNA were digested with restriction enzymes HindIII-HF and SalI-HF, which yield fragments of 4733 bp for the vector and 924 bp for the insert.

Ligation and transformation of pECFP-N1 and hZAG

The digested vector and insert DNA was cut out of the gel and purified again for ligation. After transformation of competent *E. coli* DH5α cells, a few colonies were digested with the high-fidelity restriction enzymes SpeI-HF and NotI-HF to check for correct integration of the ZAG insert. Restriction digest with these enzymes should yield two fragments with a size of 4861 bp and 767 bp if the insert is correctly integrated into the vector. This can be seen in figure 4.6. To ensure that there are no mutations, again one of the clones was sent for sequencing to Microsynth Austria GmbH and no mutations could be found (not shown).

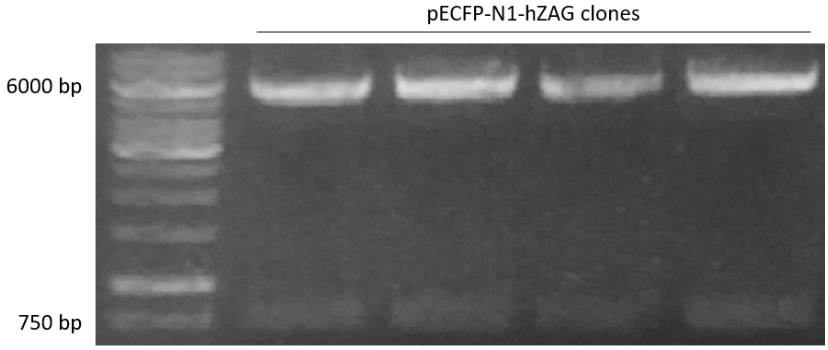

Figure 4.6: **Restriction digest of pECFP-N1-hZAG.** The used restriction enzymes SpeI-HF and NotI-HF yield DNA fragments of the size of 4861 bp and 767 bp if the insert is correctly integrated into the vector.

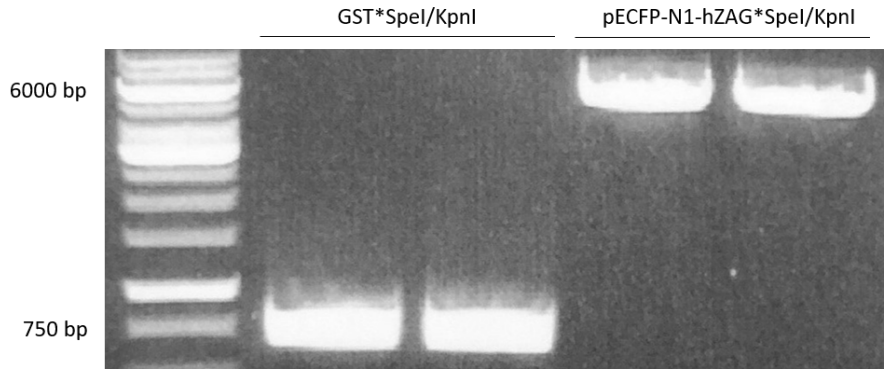

Figure 4.7: **Restriction digest of GST and pECFP-N1-hZAG.** Vector and insert DNA were digested with restriction enzymes SpeI-HF and KpnI-HF, which yield fragments of 738 bp and 5628 bp for insert and vector respectively.

Restriction digest of pECFP-N1-hZAG and GST

The next step was the cloning of the GST-tag into the ZAG-containing pECFP-N1 vector. The amplified GST insert and the vector were cut with the high-fidelity restriction enzymes SpeI-HF and KpnI-HF. The linearised ZAG-containing vector (5628 bp) and the GST insert (738 bp) are shown in figure 4.7. Again, vector and insert were cut out of the gel and purified for ligation.

Ligation and transformation of pECFP-N1-hZAG and GST

The second ligation step was performed the same way as the first one and the colonies were digested with the high-fidelity restriction enzyme NotI-HF to ensure correct integration. If both inserts are correctly integrated into the vector, the reaction should yield two DNA fragments with a size of 5568 bp and 774 bp. As you can see in figure 4.8, according bands are visible in the agarose gel. The sequencing results of the checked clone also did not show any mutations (not shown).

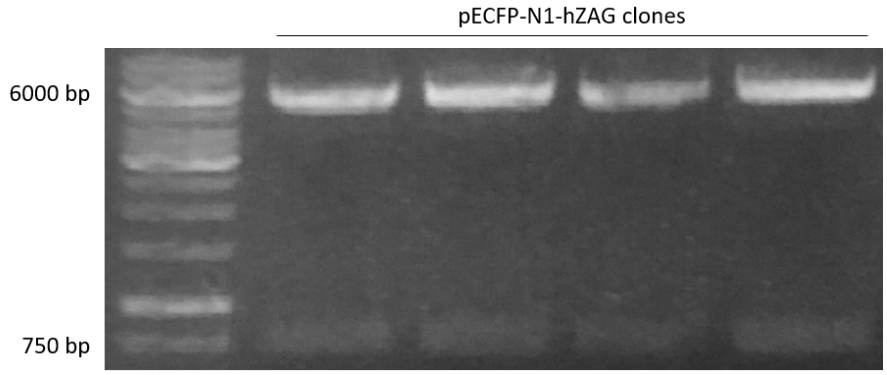

Figure 4.8: **Restriction digest of pECFP-N1-hZAG-GST.** The used restriction enzyme NotI-HF yield DNA fragments the size of 5568 bp and 774 bp if the insert is correctly integrated.

4.2 Overexpression and protein purification

To produce high amounts of His- and GST-tagged human ZAG for the protein interaction studies, the recombinant plasmids pcDNA-myc-hZAG-His-C and pECFP-N1-hZAG-GST were overexpressed in Expi293 cells. Three days after transfection, the media containing secreted ZAG was harvested and applied for protein purification by affinity chromatography as described in the methods section 3.3. Purification of both His- and GST tagged human ZAG, yielded protein concentrations up to 7,6 mg/ml (total $\approx 6-7$ mg) dependent on the the batch of Expi293 cells.

4.3 Protein interaction studies

To investigate protein-protein interactions between human ZAG and human and murine adipocyte plasma membranes, two protein interaction studies were performed. In both experiments, the biotinylated cross-linking reagent Sulfo-SBED was used. To visualize protein-protein interactions by the label transfer method, the cross-linked proteins were separated by SDS-PAGE under reducing and non-reducing conditions, followed by western blot analysis.

4.3.1 Sulfo-SBED cross-linking of human ZAG and adipocytes

In preparation for cross-linking, hMADS were differentiated for 19 to 22 days and harvested as described in the methods section 3.2.2 and 3.4.1. His- and GST-tagged ZAG as well as the controls GST and β-galactosidase were labelled with a 3-fold molar excess of Sulfo-SBED and cross-linked with whole cells and cell lysates by UV-light. After separation by SDS-PAGE under reducing and non-reducing conditions, the biotin-tag on interacting proteins was analysed by western blotting. In the very first experiment, GST-tagged hZAG was cross-linked with hMADS in 6-well plates. Three wells were used as intact cells, the other three as lysates. One well of intact cells cross-linked with ZAG was washed with 1x PBS after cross-linking. As a control non-cross-linked Sulfo-SBED labelled ZAG was used. Both blots were incubated first with an anti-ZAG antibody to detect ZAG in the samples. Due to the label transfer method there should be a shift visible in molecular weight between the non-reduced and reduced samples. Under non-reduced conditions ZAG is linked to interacting proteins, which causes a higher molecular weight (MW) compared to non-linked ZAG.
As shown in figure 4.9, ZAG could be detected in lysates, but not in the whole cell samples under reduced conditions and there seems to be a difference between the lysates. GST-tagged ZAG has a MW of approximately 66 kDa, dependent on glycosylation. The ZAG control could be detected as well, however, the band appears at a molekular weight way higher than it should. Under reduced conditions multiple bands are visible in the lysates and the non-washed samples containing whole cells cross-linked with ZAG. The most intense bands remain on the same height as under non-reducing conditions. The same blots were incubated with streptavidin-HRP to detect the biotin-tag, which should be transferred to the interaction partner under reducing conditions. As shown in figure 4.10 the non-reduced blot incubated with streptavidin-HRP looks very similar to incubation with anti-ZAG antibody. Again, there are no bands visible in the samples containing whole cells, but in the lysates. This time, the ZAG control seems to be at the right height. Under reducing conditions multiple bands are visible in all samples, except for the control. The signals in ZAG and GST controls were similar.

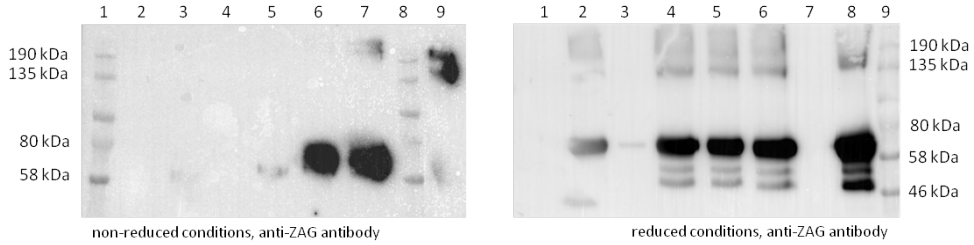

Figure 4.9: **cross-linking of hZAG with hMADS, western blot with anti-ZAG antibody.** Sulfo-SBED labelled GST-tagged hZAG was cross-linked with differentiated hMADS in 6-well plates. This was performed with intact cells as well as cell lysates. Applied samples (from left to right): non-reduced conditions 1. color prestained protein standard (broad range), 2. GST-tagged human ZAG cross-linked with intact hMADS and washed with PBS after cross-linking, 3. GST-tagged human ZAG cross-linked with intact hMADS, 3. GST cross-linked with intact hMADS, 5.-7. GST-tagged human ZAG cross-linked with cell lysates of hMADS, 8. standard, 9. GST-tagged human ZAG labelled with Sulfo-SBED; reduced conditions 1. GST-tagged human ZAG cross-linked with intact hMADS and washed with 1x PBS after cross-linking, 2. GST-tagged human ZAG cross-linked with intact hMADS, 3. GST cross-linked with intact hMADS, 4.-6. - GST-tagged human ZAG cross-linked with cell lysates of hMADS, 7. empty, 8. GST-tagged human ZAG labelled with Sulfo-SBED, 9. standard.

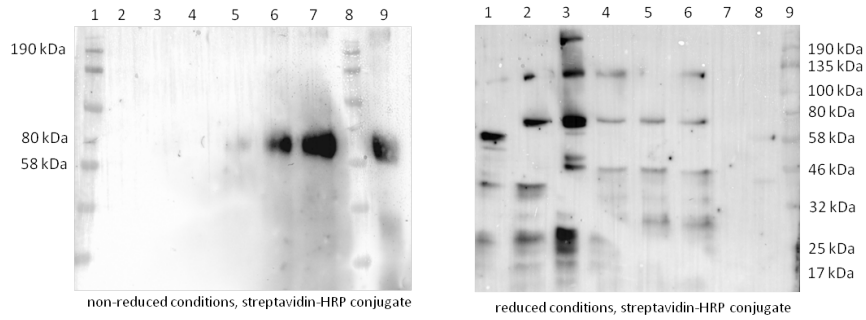

Figure 4.10: **Cross-linking of hZAG with hMADS, western blot with streptavidin-HRP.** Sulfo-SBED labelled GST-tagged hZAG was cross-linked with differentiated hMADS in 6-well plates. This was performed with intact cells as well as cell lysates. Applied samples (from left to right): non-reduced conditions 1. color prestained protein standard (broad range), 2. GST-tagged human ZAG cross-linked with intact hMADS and washed with PBS after cross-linking, 3. GST-tagged human ZAG cross-linked with intact hMADS, 3. GST cross-linked with intact hMADS, 5.-7. GST-tagged human ZAG cross-linked with cell lysates of hMADS, 8. standard, 9. GST-tagged human ZAG labelled with Sulfo-SBED; reduced conditions 1. GST-tagged human ZAG cross-linked with intact hMADS and washed with 1x PBS after cross-linking, 2. GST-tagged human ZAG cross-linked with intact hMADS, 3. GST cross-linked with intact hMADS, 4.-6. - GST-tagged human ZAG cross-linked with cell lysates of hMADS, 7. empty, 8. GST-tagged human ZAG labelled with Sulfo-SBED, 9. standard.

To eliminate putative interactions of the GST-tag, the experiment was performed again using His-tagged human ZAG. Sulfo-SBED labelled His- and GST-tagged hZAG and the labelled controls GST and β-galactosidase were cross-linked with intact hMADS in 6-well plates. The blots containing non-reduced and reduced samples were incubated first with the anti-ZAG antibody. In figure 4.11 you can see signals for ZAG-GST on its single molecular weight and higher. His-tagged ZAG is visible at a molecular weight of approximately 40 kDa, with additional signals indicated higher. In one GST sample, a band is visible at the same height as the GST-tagged ZAG, which could be overflown neighboring sample. Under non-reducing conditions multiple smaller bands are visible for both His- and GST-tagged ZAG samples. Incubated with streptavidin-HRP, the non-reduced blot shows signals for proteins of various sizes in every sample, except for the first GST sample. As shown figure 4.12, the most prominent bands in the ZAG samples are at the assumed height for ZAG itself. Under reduced conditions distinctive bands are visible in all of the samples and non of the signals seems to appear specifically in the ZAG samples.

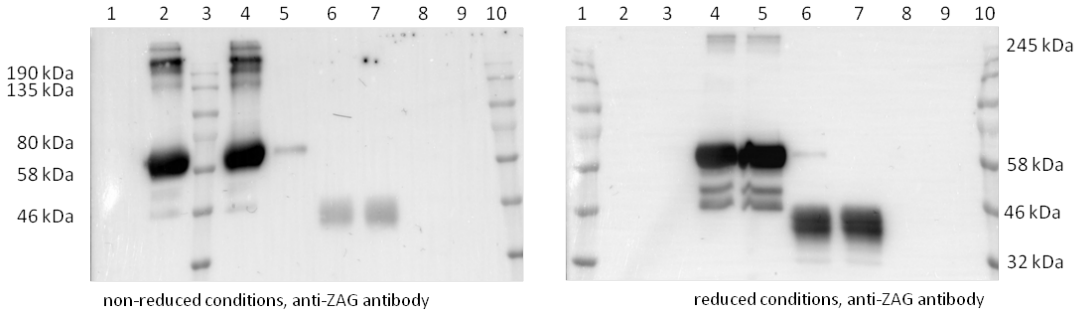

Figure 4.11: **Cross-linking of hZAG with hMADS, western blot with anti-ZAG antibody.** Sulfo-SBED labelled His- and GST-tagged hZAG was cross-linked with differentiated hMADS. As controls cells were cross-linked with labelled GST and β-galactosidase. Applied samples were all cross-linked with intact hMADS (from left to right): non-reduced conditions 1. GST, 2. GST-tagged human ZAG, 3. color prestained protein standard (broad range) 4. GST-tagged human ZAG 5. GST, 6.-7. His-tagged human ZAG, 8.-9. β-galactosidase, 10. standard; reduced conditions 1. standard, 2.-3. GST, 4.-5. GST-tagged human ZAG, 6.-7. His-tagged human ZAG, 8.-9. β-galactosidase, 10. standard.

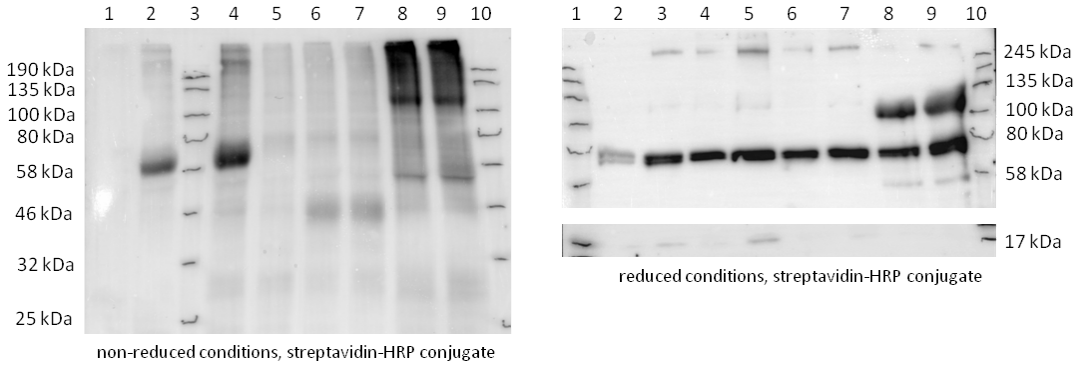

Figure 4.12: **Cross-linking of hZAG with hMADS, western blot with streptavidin-HRP.** Sulfo-SBED labelled His- and GST-tagged hZAG was cross-linked with differentiated hMADS. As controls cells were cross-linked with labelled GST and β-galactosidase. Applied samples were all cross-linked with intact hMADS (from left to right): non-reduced conditions 1. GST, 2. GST-tagged human ZAG, 3. color prestained protein standard (broad range) 4. GST-tagged human ZAG 5. GST, 6.-7. His-tagged human ZAG, 8.-9. β-galactosidase, 10. standard; reduced conditions 1. standard, 2.-3. GST, 4.-5. GST-tagged human ZAG, 6.-7. His-tagged human ZAG, 8.-9. β-galactosidase, 10. standard.

4.3.2 Plasma membrane enrichment

In order to achieve more specific signals for ZAG, the cross-linking was performed with isolated plasma membrane from mouse adipose tissue. Plasma membrane from white adipose tissue of wild type mice was isolated as described in the methods section (3.4.3) and prepared for cross-linking. The enrichment of plasma membrane, having a higher free cholesterol content than other membranes, during the isolation steps was confirmed by the enrichment of free cholesterol. In figure 4.13, an increase in free cholesterol per mg protein could be measured. In the first fraction, the 1000 x g supernatant of the homogenate, 2,68 μg free cholesterol/mg protein was measured. The second fraction represents the total membrane fraction and was taken after ultra centrifugation at 35000 rpm and already showed a free cholesterol level of 8,7 μg/mg protein. The third fraction represents the isolated plasma membrane fraction after density centrifugation on sucrose solution containing 35,76 μg free cholesterol / mg protein.

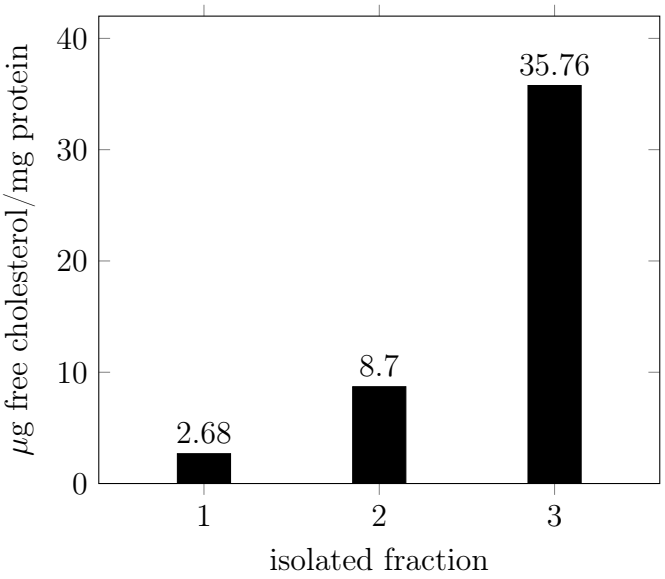

Figure 4.13: **Plasma membrane enrichment.** Isolated fractions: 1 - 1000 x g supernatant of homogenized murine white adipose tissue, 2 - pellet from 35000 rpm centrifugation, 3 - isolated plasma membrane fraction.

4.3.3 Sulfo-SBED cross-linking of human ZAG and murine adipocyte plasma membrane

His-tagged human ZAG and PEDF were labelled with Sulfo-SBED and cross-linked with isolated murine adipozyte plasma membrane. As controls, another His-tagged protein (PEDF) as well as unlabelled ZAG and PEDF were used. To reduce nonspecific label transfer, 0,2 % BSA was added to one ZAG and PEDF sample. The blot performed under non-reducing conditions was incubated with anti-His antibody and the one performed under reducing conditions with anti-ZAG antibody. Under non-reducing conditions, we observed bands with high molecular weight in every sample, as expected for the tagged proteins (figure 4.14) as well as bands at the right height for His-tagged ZAG ($\approx$ 40 kDa) in the cross-linked ZAG samples and for PEDF (50 kDa) in the unlabelled PEDF sample. The blot performed under reduced conditions incubated wit anti-ZAG antibody shows bands for ZAG at the corresponding height.

Incubation of the same blots with streptavidin-HRP revealed the same bands for ZAG, but not for PEDF compared to incubation with anti-His antibody. As shown in figure 4.15, the high MW bands visible on the anti-His incubated blot, are visible in the cross-linked ZAG samples and also in the PEDF/BSA sample. Additionally, a band slightly under the 80 kDa band of the standard is visible in all samples, except for the unlabelled ZAG and the PEDF samples. Under reduced conditions, multiple bands are visible in every sample. By looking at the reduced conditions, multiple bands are visible in every sample. These bands, with the most prominent ones slightly under the 80 kDa band of the standard and between 100 kDa and 135 kDa, are similar in all samples, which indicates unspecific signals or interaction with cell material. In summary, there is a shift in MW between non-reduced and reduced conditions, but none of the signals seems to be specific for ZAG samples compared to the controls.

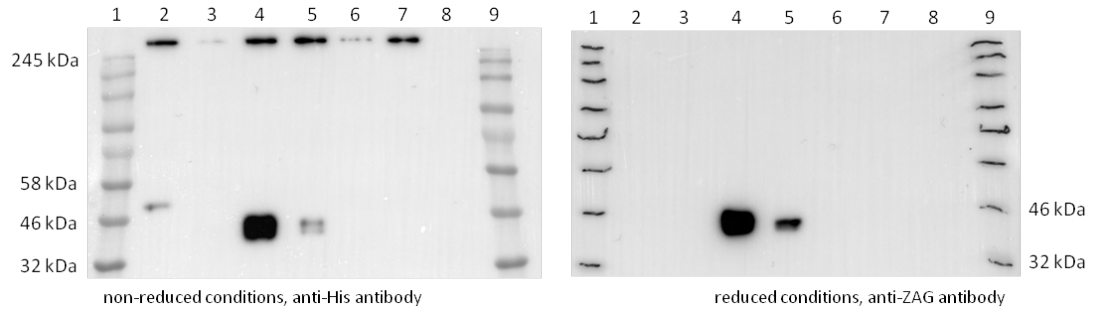

Figure 4.14: **Cross-linking of hZAG with murine adipocyte plasma membrane, western blot with anti-ZAG/His antibody.** Sulfo-SBED labelled His-tagged hZAG and PEDF were cross-linked with murine adipozyte plasma membrane. Applied samples (from left to right): on both blots 1. color prestained protein standard (broad range), 2. unlabelled PEDF, 3. unlabelled His-tagged human ZAG, 4. His-tagged human ZAG cross-linked with plasma membrane (PM), 5. His-tagged human ZAG with 0,2 % BSA cross-linked with PM, 6. PEDF cross-linked with PM, 7. PEDF with 0,2 % BSA cross-linked with PM, 8. empty, 9. standard.

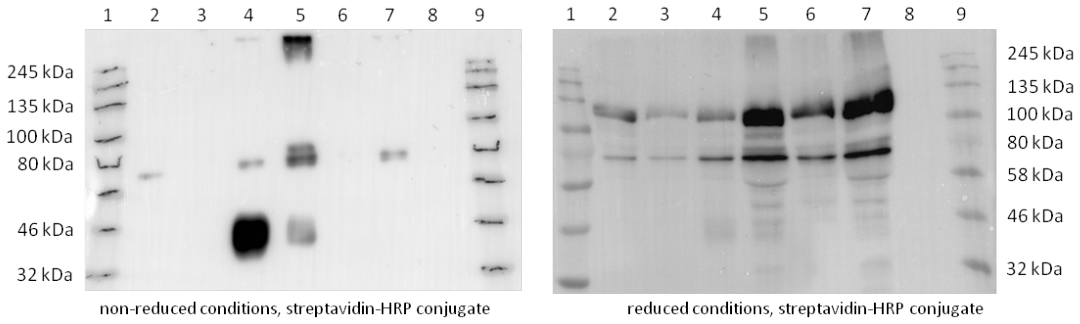

Figure 4.15: **Cross-linking of hZAG with murine adipocyte plasma membrane, western blot with streptavidin-HRP.** Sulfo-SBED labelled His-tagged hZAG and PEDF were cross-linked with murine adipozyte plasma membrane. Applied samples (from left to right): on both blots 1. color prestained protein standard (broad range), 2. unlabelled PEDF, 3. unlabelled His-tagged human ZAG, 4. His-tagged human ZAG cross-linked with plasma membrane (PM), 5. His-tagged human ZAG with 0,2 % BSA cross-linked with PM, 6. PEDF cross-linked with PM, 7. PEDF with 0,2 % BSA cross-linked with PM, 8. empty, 9. standard.

Streptravidin agarose purification after cross-linking

To increase sensitivity and reduce unspecific signals, the cross-linking experiment was performed again followed by purification of the biotinylated proteins using streptavidin agarose. The plasma membrane isolation and cross-linking were performed the same way as in the previous experiment. His-tagged hZAG was cross-linked with the isolated plasma membrane and re-purified. His-tagged PEDF and RBP4 were used as controls. The blots preformed under non-reducing and reducing conditions were incubated with streptavidin-HRP to detect the biotin-tag. Under non-reducing conditions, intense bands at approximately 40 kDa are visible in the labelled ZAG samples, as shown in figure 4.16. The labelled PEDF and RBP4 samples also show intense bands at their molecular weight of 50 kDa and 23 kDa, respectively. All labelled samples show additional signals at a higher molecular weight. Under reducing conditions, more distinct bands are visible, however, the most prominent bands remain on the height of the molecular weight of the labelled proteins, which were also visible under non-reducing conditions. The only additional bands compared to the non-reduced blot are an unspecific band slightly smaller than 80 kDa, which appears in every sample and possibly a band at 46 kDa, which is visible in the RBP4 sample. Due to the fact that the most prominent bands under reducing conditions were the same as under non-reducing

conditions and appeared even in the non-cross-linked samples, to verify reduction: another aliquot of the used cross-linked samples was reduced again with a higher amount of β-mercaptoethanol to ensure the disulfide bond of the crosslinker is reduced accurately. As shown in figure 4.17 there was little change comparing both reduced blots. The prominent bands remain the same and are visible even in the non-cross-linked labelled samples. The potentially specific band in the cross-linked ZAG sample could be detected again on this blot. This experiment suggested that the labelled proteins predominantly cross-linked with themselves.

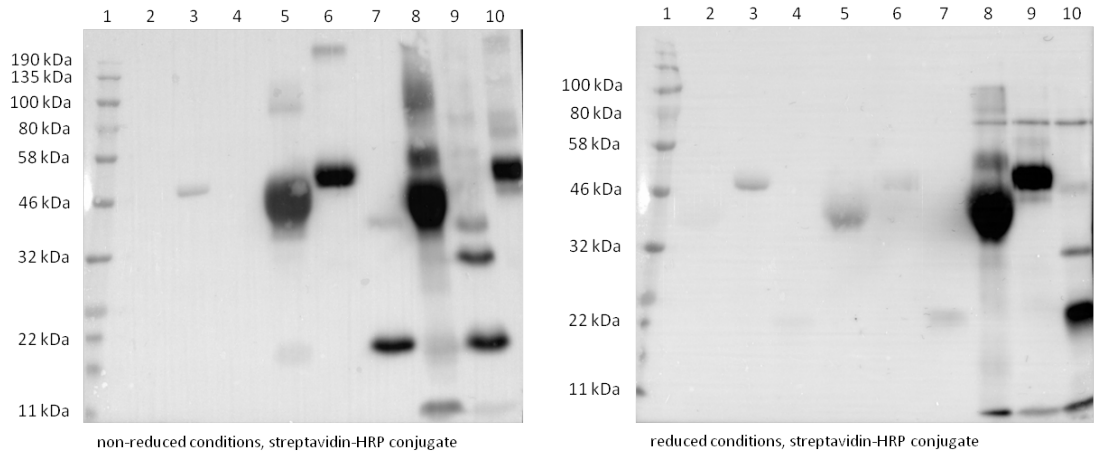

Figure 4.16: **Cross-linking of hZAG with murine adipocyte plasma membrane, western blot with streptavidin-HRP.** Sulfo-SBED labelled His-tagged hZAG, PEDF and RBP4 were cross-linked with murine adipozyte plasma membrane and purified with streptavidin agarose after cross-linking. As controls labelled and unlabelled hZAG, PEDF and RBP4 were used. Applied samples (from left to right): non-reduced conditions 1. color prestained protein standard (broad range) 2. unlabelled His-tagged human ZAG, 3. unlabelled PEDF, 4. unlabelled RBP4, 5. labelled His-tagged human ZAG, 6. labelled PEDF, 7. labelled RBP4, 8. His-tagged human ZAG cross-linked with murine plasma membrane (PM), 9. RBP4 cross-linked with murine PM 10. PEDF cross-linked with murine PM; reduced conditions 1. standard, 2. unlabelled His-tagged human ZAG, 3. unlabelled PEDF, 4. unlabelled RBP4, 5. labelled His-tagged human ZAG, 6. labelled PEDF, 7. labelled RBP4, 8. His-tagged human ZAG cross-linked with murine PM, 9. PEDF cross-linked with murine PM, 10. RBP4 cross-linked with murine PM.

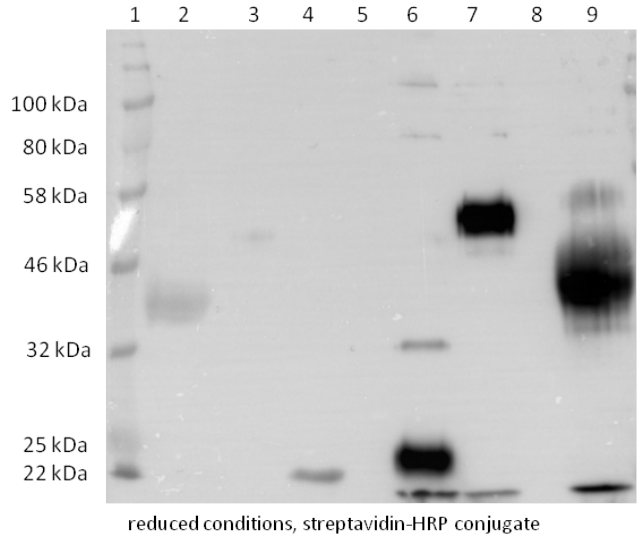

Figure 4.17: **Cross-linking of hZAG with murine adipocyte plasma membrane, western blot with streptavidin-HRP.** Sulfo-SBED labelled His-tagged hZAG, PEDF and RBP4 were cross-linked with murine adipozyte plasma membrane, purified with streptavidin agarose and reduced with 150 mM β-mercaptoethanol after cross-linking. As controls labelled hZAG, PEDF and RBP4 were used. Applied samples (from left to right): 1. color prestained protein standard (broad range) 2. labelled His-tagged human ZAG, 3. labelled PEDF, 4. labelled RBP4, 5. empty, 6. RBP4 cross-linked with murine plasma membrane (PM), 7. PEDF cross-linked with murine PM, 8. empty, 9. His-tagged human ZAG cross-linked with murine PM.

Purification of interaction partners

To test if the proteins were indeed self-conjugated during cross-linking, a last experiment was performed with the remaining aliquot of the cross-linked samples. The samples were purified again using streptavidin agarose, but this time the columns were washed with β-mercaptoethanol to remove the labelled protein from the biotinylated protein complex. The interacting proteins were bound to the streptavidin agarose slurry by the biotin-tag, which is located on the interaction partners of the labelled proteins. By reduction of the disulfide bond, the initially labelled proteins are washed out of the column and the biotinylated interaction partners stay bound to the agarose. This way the following eluate is expected to contain only the cross-linked interaction partners of ZAG, PEDF and RBP4. The interaction partners were eluted like in the previous experiments and applied for SDS-PAGE and western blotting. As shown in figure 4.18, incubation with streptavidin-HRP shows the same bands that could be detected in the previous

attempt (4.17). All three samples show bands of their single molecular weight (ZAG at ≈ 40 kDa, PEDF at 50 kDa and RBP4 at 23 kDa) and the same band slightly under the 80 kDa band of the standard. Compared to non-reducing conditions (figure 4.17) there are no additional bands visible.

In summary, purification of the biotinylated proteins after cross-linking improved the detected signals. However, ZAG, PEDF and RBP4 could be detected even after removal of the initially labelled proteins and no specific bands for interacting proteins could be found. This suggests that the proteins predominantly react with themselves.

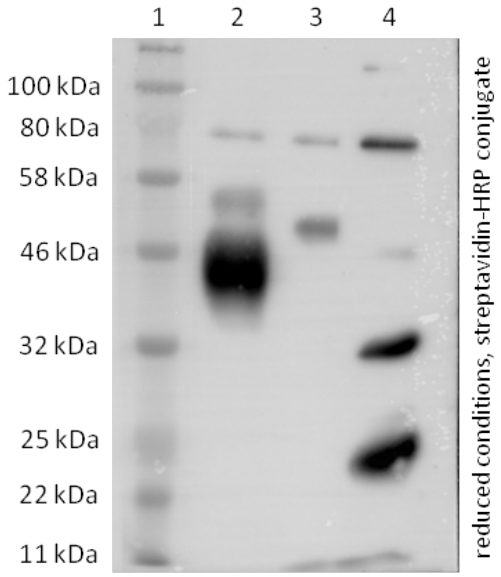

Figure 4.18: **Cross-linking of hZAG with murine adipocyte plasma membrane, western blot with streptavidin-HRP.** Sulfo-SBED labelled His-tagged hZAG, PEDF and RBP4 were cross-linked with murine adipozyte plasma membrane, reduced and purified with streptavidin agarose after cross-linking. Applied samples (from left to right): 1. color prestained protein standard (broad range), 2. His-tagged human ZAG cross-linked with murine plasma membrane (PM), 3. PEDF cross-linked with murine PM, 4. RBP4 cross-linked with murine PM.

5 Discussion

5.1 Cloning

The first goal to achieve was the cloning of two recombinant human ZAG constructs containing a Histidine or GST protein tag for purification. Since ZAG is a secreted glycoprotein it contains a N-terminal signal sequence for secretion. For this reason the protein tag had to be added to the C-terminal side of the ZAG coding sequence for proper secretion. To maintain right glycosylation of ZAG as well, it was cloned into mammalian expression vectors and overexpressed in a human cell line. Two recombinant constructs with different protein tags were designed. The coding sequence of human ZAG was cloned into the pcDNA4-myc-His-C mammalian expression vector, which adds a C-terminal His-tag to the protein. The His-tagged construct could be cloned without any problems.
For the GST-tagged construct no mammalian expression vector with a C-terminal GST-tag was available. The GST coding sequence had to be amplified from a bacterial expression vector and cloned into a mammalian expression vector together with the ZAG coding sequence. Therefore, a vector had to be found, which allows insertion of both inserts next to each other, with GST C-terminal of ZAG and has unique restriction sites not present in one of the inserts. The mammalian expression vector pECFP-N1 was found to be compatible with both inserts. Since the vector adds a C-terminal fluorescent tag, a stop codon had to be added to the GST sequence. The first attempt to ligate ZAG and GST together and insert it into the vector in a second step was not successful. The amplified sequences for ZAG and GST were correctly digested, however, no ligation product of the right size was visible in the gel. In the second approach, the GST-tagged ZAG construct could be successfully cloned into the pECFP-N1 vector in two separate cloning steps. ZAG was cloned into the vector, transformed in competent *E. coli* DH5α cells and

purified, followed by cloning of GST into the ZAG-containing vector. Therefore, specific primers had to be designed for ZAG to add two consecutive restriction sites on the C-terminal end. The outer one was used for cloning into the vector, the inner restriction site was shared with GST.

5.2 Cell culture

5.2.1 Transient overexpression in Expi293 cells

The transient overexpression of both recombinant ZAG constructs was performed in Expi293F cells. The reasons for choosing this cell line are the uncomplicated cultivation in suspension culture and the usually high protein yields. Their human origin, which enables proper glycoslylation was a reason as well. This cell line is based on suspension-adapted human embryonic kidney cells and is intended for high-yield protein expression. Since ZAG is a secreted protein it was easily purified from the media, with highest yields (up to 7,6 mg/ml) three days after transfection enhancing. After six days protein concentrations still up to 2,8 mg/ml could be purified.

5.2.2 Differentiaton of hMADS

To investigate protein-protein interactions between human ZAG and human adipocyte plasma membranes, a cross-linking experiment was performed with human multipotent adipose derived stem cells (hMADS). The cells were cultivated in special growth medium (see table 7.9, page 58) and subcultured before they reached 70 % confluence. During cultivation it was necessary to frequently check the morphology of the cells, since a change from spindle-shaped cells into large flat cells indicates a change in proliferation ability. The cells are spindle-shaped in the fast-cycling proliferation state and appear as large flat cells in slow-cycling state, which shows much slower population doubling [39]. To maintain the cells in the fast-cycling state, FGF2 was added to the growth medium in passages above 15. For differentiation of hMADS the medium was changed to different differentiation media with and without IMBX and dexamethasone as described in the methods

section 3.2.2. After seven days, adipose cells with lipid droplets became visible in bright field microscopy. The differentiation process was carried out for 19 to 22 days to the point were the cells were filled with large lipid droplets. Since the cells were very delicate upon differentiation it was necessary to handle them particularly careful. The more lipid droplets were formed, the easier the cells were detaching from the surface. The differentiation in general was not successful for every batch and it took several attempts to achieve uniformly differentiated healthy cells for the cross-linking experiments.
Due to the time consuming cultivation and differentiation of hMADS and the unsatisfying cross-linking results, the second experiment was performed with plasma membrane from mouse adipose tissue.

5.3 Protein interaction studies

5.3.1 Protein-protein interactions between ZAG and hMADS

To investigate protein-protein interactions between human ZAG and human adipozyte plasma membranes, His- and GST-tagged human ZAG was cross-linked with hMADS. In the very first attempt, GST-tagged ZAG and GST as a control were cross-linked with intact hMADS and cell lysates in 6-well plates to examine differences between cell surface and lysate interactions. The cross-linked proteins were separated under reducing and non-reducing conditions on 10 % SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. By incubation with streptavidin, a label transfer of the biotin-tag added by cross-linking, from labelled ZAG to an interacting protein should have been detected as a shift in MW. By incubation of the blots with streptavidin-HRP, such a shift could be detected under reducing conditions (figure 4.10, page 37). The band patterns of GST-tagged ZAG and GST look similar and cross-linking of intact cells and cell lysates does not show any differences. This indicates, that the signals detected on the blot are the results of unspecific interactions with cell material or interactions formed by the GST-tag instead of ZAG. On the blot performed under non-reducing conditions, no bands could be detected for ZAG and GST samples cross-linked with intact cells. Also,

the lysate triplicates seem to be different from each other. The same blot incubated with a different antibody shows the same pattern (figure 4.9, page 37). On the left side of the blot performed under non-reducing conditions no signal could be detected in the first three lanes and the lysate triplicates differ from each other. This indicates that there might have been a problem in the blotting process, where the samples on the left side of the blot were not transferred onto the membrane.
To eliminate putative interactions of the GST-tag, the experiment was repeated with His-tagged human ZAG and GST as well as β-galactosidase as controls. As shown in figure 4.12 under reducing conditions, a shift in MW is visible again. However, non of the signals was detected specifically in the ZAG samples. The most intense signals were detected in all samples and therefore seem to be unspecific.

5.3.2 Protein-protein interactions between ZAG and murine adipocyte plasma membrane

To reduce unspecific interactions with cell material, further experiments were performed with isolated plasma membrane from mouse adipose tissue instead of intact adipocytes. His-tagged human ZAG was cross-linked with isolated plasma membrane in the same way as in the first experiment. As controls, the His-tagged proteins PEDF and RBP4 were used. In the first approach ZAG and PEDF were corsslinked with the plasma membrane. To satisfy unspecific interactions, 0,2 % BSA was added to one sample of ZAG and PEDF. The proteins were separated under reducing and non-reducing conditions on 10 % SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. As shown in figure 4.15 on page 42, the samples show a shift in MW under reducing conditions when incubated with streptavidin-HRP. Like in the previous experiment, the most intense bands are visible in all samples and seem to be unspecific interactions, since these bands occur even in the unlabelled cross-linked samples. ZAG does not show any specific signals compared to the controls.
To increase the sensitivity and reduce unspecific signals, the cross-linked proteins were re-purified with streptavidin agarose in the following approach. This way, the purified samples should contain only proteins and protein complexes tagged

with biotin. As shown in figure 4.17 on page 44, His-tagged human ZAG and the controls PEDF and RBP4 show a similar pattern under non-reducing and reducing conditions. On both blots the prominent bands are detected at the MW of ZAG ($\approx$ 40 kDa), PEDF (50 kDa) and RBP4 (23 kDa). A shift in MW is visible under reducing conditions, all cross-linked samples show an additional band slightly under the 80 kDa band of the standard, which was previously indicated as unspecific. In the RBP4 sample, an additional band at 46 kDa could be detected. The ZAG sample does not show any specific bands. The signals at the MW of each labelled protein could also be detected in the non-cross-linked samples, which indicates potential self-conjugation.

To verify if the proteins are actually self-conjugating during the process, the already purified cross-linked samples were purified a second time using streptavidin agarose. The samples were bound to the slurry and washed with β-mercaptoethanol to remove the initially labelled proteins ZAG, PEDF and RBP4 by reduction of the disulfide bond. The interaction partners carrying the biotin-tag should stay bound to the slurry and were eluted like in the previous attempt. After SDS-PAGE and western blotting, the same band pattern could be detected as in the previous experiment. As shown in figure 4.18 on page 45, the most prominent bands remain on the MW of ZAG, PEDF and RBP4, in all samples the previously detected band slightly under the 80 kDa band of the standard is visible as well. No additional bands could be detected in comparison with the non-reduced conditions. This indicates that the proteins are predominantly self-conjugating in this cross-linking experiment. It is possible, that other interaction partners, which have a MW close to the MW of ZAG, could not be detected, due to the intense signal of ZAG itself. For example, in a study published by Russell, Hirai and Tisdale in 2002, it is suggested, that ZAG interacts with β3-adrenergic receptor (β3-AR) [15]. β3-AR has a MW of 43 kDa, which could be masked by the intense signal of ZAG at $\approx$ 40 kDa.

In summary, no specific interaction partners of ZAG could be found in human adipocyte membranes of hMADS and isolated murine plasma membrane through cross-linking with Sulfo-SBED. Purification with streptavidin agarose after cross-linking does increase specificity. By the use of the label transfer method only self conjugation of ZAG could be detected, indicating the formation of di- or oligomers. However, potential interaction partners in the same MW rage as ZAG might be masked by the intense ZAG signal.

6 Outlook

The aim of the master thesis was to find specific protein-protein interactions of human ZAG and adipocyte plasma membranes. Under the applied conditions no specific interactions could be found. The tendency of ZAG to predominantly self-conjugate under this conditions and its intense signal could mask potential interaction partners in the same MW range. Therefore, it is necessary to perform further experiments to achieve better reaction conditions. A future approach might be to optimize protein and crosslinker concentrations, which could suppress self-conjugation. Another approach would be to use a different cross-linking reagent with more specific conjugation abilities.

7 Appendix

7.1 Buffers and media

In the following tables, the composition of all used buffers, solutions, bacterial growth media and cell culture media is listed.

7.1.1 Buffers and other solutions

Table 7.1: **Phosphate buffered saline (PBS)** 10x stock solution

Component	**Concentration**
NaCl	1,37 M
KCl	27 mM
Na_2HPO_4 x H_2O	43 mM
KH_2PO_4	14 mM
pH:	7,3 - 7,4

Table 7.2: **Tris-Acetate-EDTA (TAE)** 50x stock solution

Component	**Amount for 1 L**
Tris	242 g
Acetate (100 %)	57,1 ml
EDTA (0,5 M)	100 ml
pH:	8,0

Table 7.3: **SDS sample buffers.** 4x reducing and non-reducing sample buffer

Component	**reducing**	**non-reducing**
Tris-HCl	0,2 M	0,25 M
SDS	8 %	8 %
Glycerol	40 %	40 %
Bromophenol blue	6 mM	0,02 %
DTT	0,4 M	-

Table 7.4: **Composition of 10 % SDS gel for SDS-PAGE**

Components for 10 ml	**separating gel**	**stacking gel**
ddH_2O	10 ml	5,9 ml
4x LGB (1,5 M Tris pH 8,8)	4,1 ml	-
4x UGB (0,5 M Tris pH 6,8)	-	2,5 ml
30 % Acrylamide	3,3 ml	1,5 ml
10 % SDS	100 μl	100 μl
TEMEND	9 μl	13 μl
10 % APS	27 μl	40 μl
0,5 % blue dye	-	30 μl

Table 7.5: **Coomassie brilliant blue staining solution**

Component	**Concentration**
Coomassie Blue	0,25 %
Acetic acid (80 %)	7,5 %
Ethanol	50 %

Table 7.6: **Destaining solution**

Component	**Concentration**
Methanol	30 %
Acetic acid (80 %)	10 %

7.1.2 Bacterial growth media

Table 7.7: **Composition of LB-Agar plates.**

Components	Concentrations
Peptone from casein	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L
Agar-agar	15 g/L
Ampicillin/Kanamycin	0,1 g/L

Table 7.8: **Composition of LB-Medium.**

Components	Concentrations
Peptone from casein	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

7.1.3 Cell culture media

Table 7.9: **Growth medium** for human multipotent adipose derived stem cells (hMADS)

Component	Concentration
DMEM Low Glucose	-
Glutamine	1x
Hepes Buffer	10 mM
Penicillin-Streptomycin	5000 IU/ml
fetal bovine serum	10 %
FGF2	2,5 ng/ml

Table 7.10: **Differentiation medium** for human multipotent adipose derived stem cells (hMADS)

Component	Concentration
DMEM/Ham's F12 (50/50)	-
Insulin	5 μg/ml
Transferrin	10 μg/ml
T3	0,2 nM
Rosiglitazone	1 μM
IBMX	100 μM
Dexamethasone	1 μM

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