

A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization

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Impairments in adiponectin multimerization lead to defects in adiponectin secretion and function and are associated with diabetes, yet the underlying mechanisms remain largely unknown. We have identified an adiponectin-interacting protein, previously named GST-kappa, by yeast 2-hybrid screening. The adiponectin-interacting protein contains 2 thioredoxin domains and has very little sequence similarity to other GST isoforms. However, this protein shares high sequence and secondary structure homology to bacterial disulfide-bond A oxidoreductase (DsbA) and is thus renamed DsbA-like protein (DsbA-L). DsbA-L is highly expressed in adipose tissue, and its expression level is negatively correlated with obesity in mice and humans. DsbA-L expression in 3T3-L1 adipocytes is stimulated by the insulin sensitizer rosiglitazone and inhibited by the inflammatory cytokine TNF α . Overexpression of DsbA-L promoted adiponectin multimerization while suppressing DsbA-L expression by RNAi markedly and selectively reduced adiponectin levels and secretion in 3T3-L1 adipocytes. Our results identify DsbA-L as a key regulator for adiponectin biosynthesis and uncover a potential new target for developing therapeutic drugs for the treatment of insulin resistance and its associated metabolic disorders.

obesity | yeast 2-hybrid system | adipose tissue | insulin resistance

Adiponectin is an adipocyte-derived hormone that plays an important role in the regulation of lipid and glucose metabolism. Adiponectin stimulates fatty acid oxidation, suppresses hepatic gluconeogenesis, increases insulin sensitivity, and acts to counter the effects of the inflammatory cytokine TNF α . Adiponectin has also been shown to have antiatherogenic effects and to act on the central nervous system to stimulate energy expenditure. Thus, adiponectin is a strong candidate for the development of drugs to treat obesity, insulin resistance, type 2 diabetes, and atherosclerosis (for review, see refs. 1–3).

Adiponectin circulating in serum exists primarily in 3 main species: a low-molecular-mass (LMM) trimer of \approx 67 kDa, a hexamer of \approx 140 kDa, and a high-molecular-mass (HMM) multimer of $>$ 300 kDa (4–6). The interaction between the collagenous domains results in formation of highly ordered trimer, which is further stabilized by an intratrimer disulfide bond mediated by Cys³⁹ (or Cys²², if the N-terminal 17-aa secretory peptide is excluded). The formation of a disulfide bond between 2 trimers mediated by the free Cys³⁹ in each leads to the formation of the hexameric form of adiponectin, serving as the building block for the HMM form, which consists of 12–18 hexamers existing in a bouquet-like structure (7). Adiponectin mutants with impaired multimerization are defective in both secretion and function and are associated with diabetes and hypoadiponectinemia (4, 6). More importantly, it has been shown that adiponectin oligomer distribution, rather than its absolute levels, correlates with thiazolidinedione-mediated increase in insulin sensitivity (8).

A number of studies have shown that adiponectin multimers are stable and do not interconvert from 1 species to another once secreted into the serum (4, 5), indicating that adiponectin multimerization is tightly regulated inside adipocytes. Several factors have

been found to affect adiponectin gene expression and secretion. For example, TNF α and IL-6 have been shown to down-regulate adiponectin gene expression and secretion (9). The plasma HMM form of adiponectin has also been shown to be suppressed by insulin and high levels of glucose (4). Conversely, the HMM adiponectin level is up-regulated by moderate weight reduction (10) or by treatment with thiazolidinediones (TZDs), which stimulate peroxisome proliferator activated receptor gamma (PPAR gamma) activity (8, 11). However, the molecules that mediate these metabolic or pharmacological effects on adiponectin disulfide bond formation and multimerization remain unknown. Because multimer complex distribution plays a major role in adiponectin's insulin sensitizing function (8), understanding the mechanisms regulating adiponectin multimerization will provide important information on the development of therapeutic interventions to treat obesity and type 2 diabetes.

Results

Identification of GST-Kappa/Disulfide-Bond A Oxidoreductase-Like Protein (DsbA-L) as an Adiponectin Interactive Protein. To identify proteins that interact with adiponectin, we screened a yeast 2-hybrid cDNA library derived from human fetal brain, using the full length mouse adiponectin without the N-terminal signal peptide sequence (amino acids 17–247) as bait. We identified 11 independent clones, including the cDNA encoding the C terminus of AdipoR1 (12) and a cDNA encoding the C terminus of a protein previously named GST-kappa (amino acid 188–216) (Fig. 1A). GST-kappa is a 25-kDa protein initially classified as a class theta GST (GST13–13) (13). Analysis of the complete amino acid sequence of the protein reveals that GST-kappa has very little sequence similarity to any other class of GST (14) but shares high sequence and secondary structure homology to *Escherichia coli* DsbA (15). Crystal structure studies also revealed that GST-kappa has the same general folding as the DsbA (16). Like other eukaryotic protein disulfide isomerases (PDIs), GST-kappa contains 2 thioredoxin-like domains (Fig. 1A). However, the GST-kappa dimer has a very different overall shape when compared with the canonical GST fold (16). For these reasons, GST-kappa has been acknowledged to be misnamed in the protein sequence database and not a member of the GST gene family (15). Based on these findings and our results showing that GST-kappa plays a key role in regulating adiponectin multimerization (see below), we renamed GST-kappa to DsbA-L.

To test whether DsbA-L interacts directly with adiponectin, we

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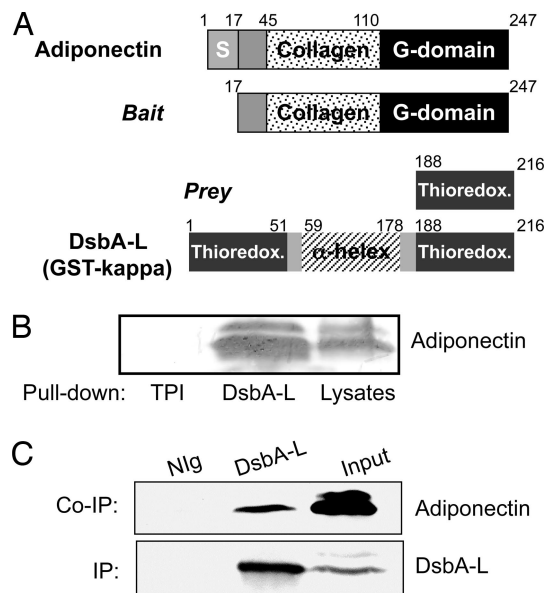


Fig. 1. Identification of GST-kappa/DsbA-L as an adiponectin interacting protein. (A) Yeast 2-hybrid screening to identify DsbA-L as an adiponectin interactive protein. The signal sequence (S), collagen and globular (G) domains of adiponectin and the thioredoxin domain and alpha-helix region of DsbA-L are indicated. (B) DsbA-L interacts with adiponectin *in vitro*. 3T3-L1 adipocyte lysates were incubated with the His-tagged control protein triosephosphate isomerase (TPI) or His-tagged DsbA-L bound to Ni-NTA-agarose beads. The bound adiponectin was detected with an anti-adiponectin antibody. (C) Interaction of adiponectin with DsbA-L in 3T3-L1 adipocytes. Endogenous DsbA-L was immunoprecipitated with a homemade antibody to the protein. Coimmunoprecipitated adiponectin was detected by Western blot with an antiadiponectin antibody. Data are representatives of 3 experiments with similar results.

expressed DsbA-L in bacterial cells and carried out *in vitro* binding studies. We found that endogenous adiponectin interacted with His-tagged DsbA-L but not with the nonrelevant control protein (Fig. 1*B*). Coimmunoprecipitation experiments revealed that endogenous adiponectin interacted with endogenous DsbA-L in 3T3-L1 adipocytes (Fig. 1*C*).

Metabolic and Pharmacological Regulation of DsbA-L Expression.

DsbA-L transcript has been shown to be ubiquitously expressed in various human (17) and mouse (18) tissues. However, whether this protein is expressed in adipose tissue has not been investigated. Because adiponectin is expressed mainly in adipocytes, we examined the expression of DsbA-L in this tissue. We found that DsbA-L is expressed in a number of mouse tissues such as liver, kidney, pancreas, heart, and lung (Fig. 2*A*). Interestingly, the highest DsbA-L expression was observed in white adipose tissue (WAT) (Fig. 2*A*), where adiponectin is synthesized and secreted. We also found that DsbA-L is highly expressed in brown adipose tissue [supporting information (SI) Fig. S1]. This finding is interesting, because adiponectin has been detected in brown adipose tissue and has been implicated to play an important role in glucose and lipid metabolism during the perinatal period (19). Consistent with previous findings (20), we found that adiponectin expression was greatly induced during 3T3-L1 cell differentiation (Fig. 2*B Top*). 3T3-L1 cell adipogenesis also led to a marked increase in the expression levels of DsbA-L (Fig. 2*B Middle*). The findings that DsbA-L is highly expressed in adipose tissue and its expression is induced during 3T3-L1 cell differentiation suggest a role of this protein in adipocyte function.

Adiponectin levels are stimulated by PPAR gamma agonists thiazolidinediones. However, adiponectin levels negatively correlate with TNF α release by adipose tissue (21). To obtain further

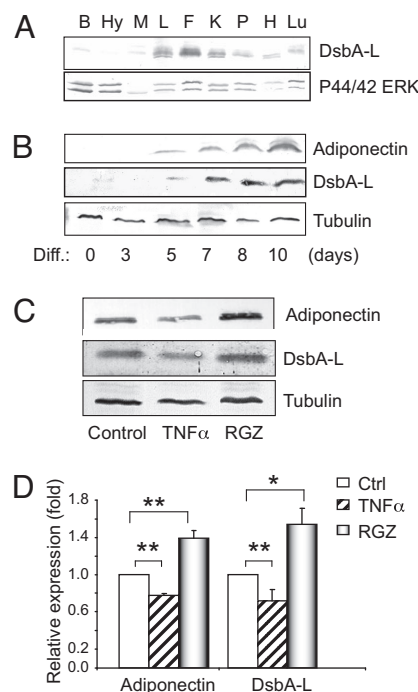


Fig. 2. Tissue distribution and regulation of DsbA-L protein expression. (A) Tissues from 4-month-old male C57BL/6J mice were homogenized and the expression of DsbA-L and ERK1/2 (as a loading control) was determined by Western blot by using indicated antibodies. B, brain; Hy, hypothalamus; M, skeletal muscle, L, liver; F, fat; K, kidney; P, pancreas; H, heart; Lu, lung. (B) DsbA-L expression is increased during 3T3-L1 cell differentiation. The expression levels of several adipokines and tubulin at different days during 3T3-L1 differentiation was determined by Western blot by using specific antibodies as indicated. (C) DsbA-L expression in 3T3-L1 adipocytes is inhibited by TNF α and stimulated by rosiglitazone (RGZ). Lysates from 3T3-L1 adipocytes treated with 10 μ M RGZ or 10 ng/ml TNF α for 24 h were analyzed by Western blot. (D) Quantification of adiponectin and DsbA-L levels from cells treated with TNF α or RGZ. Data are present as means \pm SEM from 3 independent experiments. Comparison between groups was made by 1-way ANOVA. **, $P < 0.01$; *, $P < 0.05$.

evidence on the potential role of DsbA-L in regulating adiponectin function, we investigated whether DsbA-L expression levels are regulated by these reagents. We found that the expression levels of both adiponectin and DsbA-L were stimulated by rosiglitazone (RGZ) in 3T3-L1 adipocytes. However, TNF α suppressed adiponectin and DsbA-L expression (Fig. 2*C* and *D*).

Overexpression of DsbA-L Promotes Adiponectin Multimerization and Increases Its Cellular Levels.

To determine the potential role of DsbA-L, we transiently expressed DsbA-L in differentiated 3T3-L1 CAR cells by adenovirus-mediated infection. Differentiated 3T3-L1 CAR cells stably express the truncated coxsackievirus and adenovirus receptor (CAR) and are \approx 100-fold more efficient for adenovirus infection compared with 3T3-L1 adipocytes (22). At differentiation day 9, >90% of the 3T3-L1 CAR cells were differentiated into adipocyte as determined by Oil Red O staining (Fig. S2*A Top*). In addition, high efficiency of adenovirus-mediated infection (\approx 80%) was achieved as demonstrated by GFP expression (Fig. S2*A Bottom*). Overexpressing DsbA-L in 3T3-L1 CAR adipocytes increased the cellular protein but not the mRNA levels of adiponectin (Figs. 3*A* and S2*B*). Gel filtration experiments revealed that overexpressing DsbA-L markedly increased the ratio of HMM form to LMM (total hexamer and trimer) of adiponectin in both the cell lysates and the cell culture medium of DsbA-L-overexpressing cells (Fig. 3*B–E*).

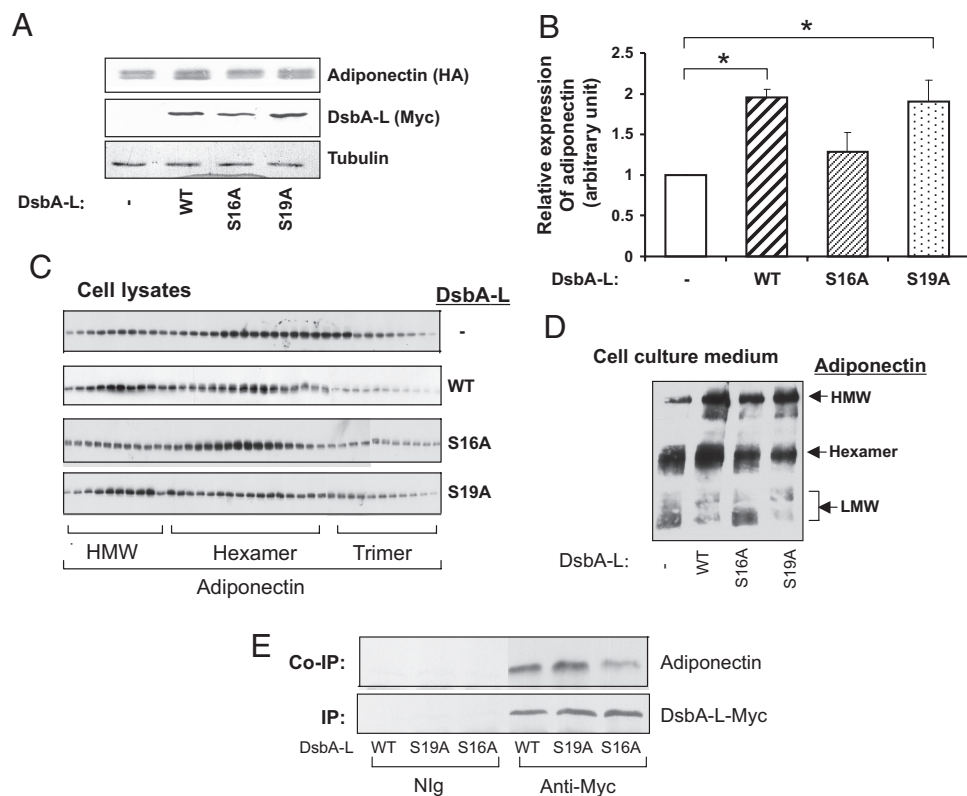


Fig. 5. Ser¹⁶ of DsbA-L plays a critical role in regulating adiponectin stability and multimerization. A mammalian expression vector encoding HA-tagged adiponectin was transfected into CHO/R cells together with a plasmid encoding myc-tagged wild-type, S16A, or S19A mutant of DsbA-L. (A) The expression of these proteins in cell lysates was determined by Western blot by using antibodies as indicated. (B) Semiquantitation of adiponectin levels by analyzing the Western blot data from 3 independent experiments as described in A by using the National Institutes of Health IMAGE program. All data were expressed as means \pm SEM. **, $P < 0.01$; *, $P < 0.05$. (C) Adiponectin oligomers in cell lysates were separated by gel filtration and determined by Western blot by using an anti-adiponectin antibody. (D) Adiponectin oligomers in cell culture medium were separated by nonreducing gel electrophoresis and determined by Western blot with an antiadiponectin antibody. Data are representatives of 3 independent experiments with similar results. (E) HA-adiponectin and myc-tagged wild-type or mutants of DsbA-L were transiently expressed in CHO/R cells. HA-tagged adiponectin coimmunoprecipitated with myc-tagged DsbA-L was detected by Western blot with an antibody to the HA-tag.

a major role in DsbA-L activity. Coimmunoprecipitation experiments showed that mutation at Ser¹⁶, but not Ser¹⁹, markedly inhibited DsbA-L binding to adiponectin (Fig. 5E). In addition, overexpression of DsbA-L neither enhanced the cellular levels of the C39S mutant of adiponectin, which is defective in forming disulfide bond (Fig. S4C) nor had any effect on the mutant adiponectin multimerization (Fig. S4D). This indicates that disulfide bond formation and multimerization is essential for DsbA-L-mediated increase in cellular levels of adiponectin.

Expression Level of DsbA-L in Adipose Tissue Negatively Correlates with Obesity in Mice and Human Subjects. Adiponectin expression is reduced in both human obesity and mouse models of genetic obesity (26). To determine whether obesity affects DsbA-L expression, we

examined the protein levels of adiponectin and DsbA-L in the epididymal WAT of normal diet (ND)-fed or high-fat-diet (HFD)-fed C57BL/6 mice (Table S4). We found that HFD feeding significantly reduced the expression levels of both adiponectin and DsbA-L (Fig. 6A and B). To confirm the clinical relevance of the above findings, we examined the expression levels of DsbA-L in the s.c. fat of 23 lean and 12 obese human subjects (Table S2). Real-time PCR experiments revealed that the mRNA levels of DsbA-L in overweight/obese subjects were significantly lower than that in lean controls ($P < 0.01$, Fig. 6C). There is significant inverse correlation between the adipose tissue DsbA-L mRNA levels and body mass index (BMI) (Fig. 6D). Furthermore, there is a strong positive correlation between DsbA-L mRNA levels in s.c. fat and serum levels of adiponectin in these individuals (Fig. 6E). We also

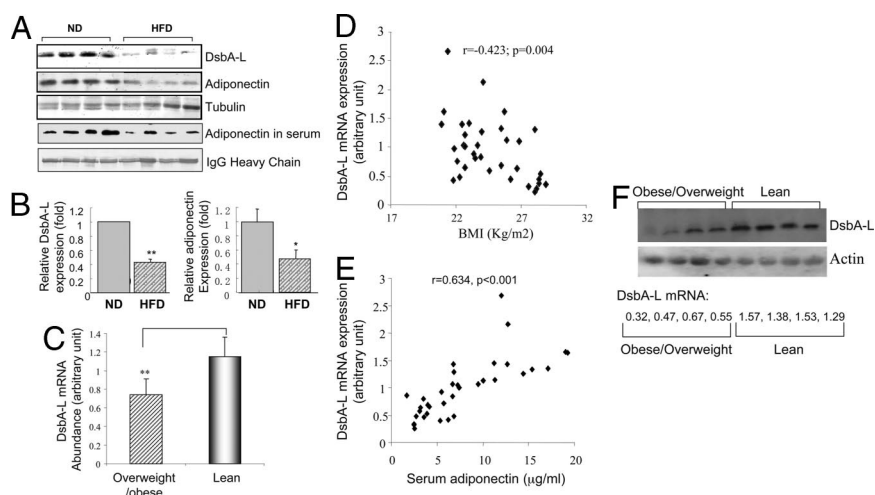


Fig. 6. DsbA-L expression levels are reduced in obese mice and humans. (A) Twenty micrograms of proteins isolated from adipose tissues of ND- or HFD-fed male C57BL/6 mice were separated by SDS/PAGE and detected by Western blot with indicated antibodies. (B) Quantitation of the relative levels of DsbA-L or adiponectin expression in adipose tissues from normal chow diet and HFD-fed mice. The fold change was determined by analyzing Western blots by using the National Institutes of Health IMAGE program and was normalized for the amount of tubulin expression. (C) Comparison of DsbA-L mRNA expression in the s.c. fat between overweight/obese (BMI >23 kg/m², $n = 12$) and lean individuals (BMI <23 kg/m², $n = 23$) (Table S2). Correlation between DsbA-L mRNA expression and BMI (D) or serum levels of adiponectin E in 35 human subjects. (F) The protein expression of DsbA-L in adipose tissues of lean (BMI 19 ± 2 ; $n = 4$) and overweight/obese (BMI 29.5 ± 2.5 ; $n = 4$) human subjects (Table S3) were examined by Western blot with an antibody to DsbA-L. Equal amounts of proteins (40 μ g) were loaded per lane. The relative mRNA levels of DsbA-L in each individual are shown.

examined the protein expression levels of DsbA-L in the s.c. fat of 4 randomly selected lean and 4 obese/overweight human subjects by Western blot analysis. Consistent with reduced DsbA-L mRNA levels, this preliminary study showed that protein levels of DsbA-L are also greatly reduced in fat tissues of overweight/obese human subjects compared with lean human subjects (Fig. 6F).

Discussion

Data accumulated over the past several years provide strong evidence that defects in the regulation of adiponectin multimerization and secretion play important roles in obesity, insulin resistance, and type 2 diabetes. Several proteins have recently been shown to regulate adiponectin trafficking and secretion (27–29). However, the mechanisms regulating adiponectin multimerization and secretion remain largely unknown.

In the present study, we present evidence showing that DsbA-L plays a critical and selective role in regulating adiponectin biosynthesis. Consistent with previous findings, we found that the secretion and expression levels of adiponectin were stimulated by rosiglitazone and inhibited by TNF α ; the latter has been shown to reduce insulin sensitivity in cells (30, 31). Furthermore, our present study suggests that DsbA-L plays a role in mediating the stimulatory effect of rosiglitazone on adiponectin biosynthesis and secretion. One possible mechanism by which suppressing DsbA-L affects rosiglitazone-stimulated adiponectin secretion is that reducing the expression levels of DsbA-L inhibits adiponectin multimerization, which is critical for secretion. Consistent with this view, adiponectin mutants unable to form HMM multimers have been shown to be defective in secretion from cells (6).

In addition to impaired secretion, suppressing DsbA-L cellular levels by RNAi also greatly reduced the cellular levels of adiponectin. Interestingly, suppressing DsbA-L had no significant effect on the mRNA levels of adiponectin (Fig. S2D), suggesting that reduced biosynthesis and/or accelerated degradation may play a major role in reducing cellular levels of adiponectin. One possibility is that a deficiency in DsbA-L expression increases the levels of unfolded or misfolded adiponectin, leading to unfolded protein response (UPR). UPR has been shown to attenuate general translation and up-regulate ER-associated protein degradation via activation of the PKR-like eukaryotic initiation factor 2 α kinase (PERK), the activating transcription factor-6 (ATF6), and the inositol requiring enzyme-1 (IRE-1) signaling pathways. UPR has also been shown to increase disposal of misfolded proteins via the ubiquitin proteasome-dependent ER-associated degradation (ERAD) pathway (32, 33). Suppression of DsbA-L expression in cells may thus reduce adiponectin levels by activating the UPR-stimulated pathways. We are currently investigating this possibility.

How DsbA-L regulates adiponectin multimerization remains to be elucidated. One possibility is that DsbA-L functions as a PDI to regulate adiponectin disulfide bond formation, which is essential for multimerization. First, DsbA-L shares high sequence and secondary structure homology to bacterial DsbA (15, 16), which catalyze disulfide bond formation during the folding of secreted proteins in bacteria (34). Like other eukaryotic PDIs, DsbA-L contains 2 thioredoxin-like domains (Fig. 1). Interestingly, DsbA-L contains a SXXS sequence (Ser¹⁶ and Ser¹⁹), which is localized in place of the classic “CXXC” redox active motif found in DsbA and TspG, a DsbA homolog in the pathogenic bacterium *Vibrio cholerae* responsible for the folding, maturation, and secretion of virulence factors (35) (Fig. S4A). It becomes evident that not all PDIs contains the classic contain classical CXXS motifs. For example, the testis-specific protein disulfide isomerase-like protein (PDILT) contains an SXXS and an SXXC motif (24). The 2 monocysteine thioredoxins in *Arabidopsis thaliana* contain the CXXS motif and are very efficient as disulfide isomerases (36). Ser¹⁶ and Ser¹⁹ of DsbA-L are extremely conserved among all species examined (Fig. S4B), suggesting an important function for these residues. Consistent with this, X-ray crystal structure studies revealed that DsbA-L

has the same general fold as DsbA and promotes formation of the thiolate E:GS⁻ through interaction of the hydroxyl group of Ser¹⁶ with the sulfur of reduced glutathione (GSH) (16). Furthermore, a mutant human PDI-related protein (hPDIr) with all of its active-site cysteines mutated to serine retained 57% of the oxidative refolding activity of wild-type enzyme in the reactivation of α 1-antitrypsin (37). In agreement with these findings, we found that replacing Ser¹⁶ with alanine disrupted the ability of DsbA-L in promoting adiponectin trimer to higher order multimers in cells (Fig. 5). Alternatively, DsbA-L may play an indirect role in facilitating adiponectin disulfide bond formation. Consistent with this view, we found that incubation with DsbA-L alone was insufficient to promote adiponectin multimerization *in vitro* (data not shown), suggesting that additional factors may be necessary for adiponectin multimerization in intact cells. It has been shown that \approx 29% of adenovirus-mediated overexpressed adiponectin are in HMM form in the liver, suggesting that hepatocytes express the intracellular machinery necessary for producing the HMM species (38). Our results showed that DsbA-L is highly expressed in the liver (Fig. 2A), which is consistent with a role of DsbA-L in regulating adiponectin multimerization. These findings suggest the regulation of adiponectin multimerization is cell type-specific, probably because of the presence or absence of specific cofactors. Further studies will be needed to test these possibilities.

It is well established that different forms of the adiponectin multimers, whose cellular levels vary in response to changes in metabolic and disease states, have distinct functions in metabolism (21, 39). In fact, the ratio of HMM forms of adiponectin to the total adiponectin level, rather than just total adiponectin level itself, has been shown to correlate more significantly with key features of central obesity and insulin resistance status (8). Our study identifies DsbA-L as a critical regulator of adiponectin multimerization and uncovers a potential therapeutic target for the diagnosis and treatment of insulin resistance and its associated diseases.

Materials and Methods

Cell Culture, Transfection, *In Vitro* Binding, Immunoprecipitation, and Western Blot. The maintenance and/or differentiation of CHO/IR and 3T3-L1 cells were performed as described in our previous studies (40). Cell transfection was performed by using Lipofectamine Plus (Invitrogen). Cell lysates and tissue homogenates were prepared in ice-cold lysis buffer, and the general procedures used for coimmunoprecipitation, *in vitro* binding and Western blot have been described (41). Additional information is provided in the *SI Methods*.

Yeast 2-Hybrid cDNA Library Screening. The adiponectin bait plasmid was constructed by subcloning a cDNA fragment encoding mouse adiponectin without the signal peptide (amino acids 13–244) into the pLexA 2-hybrid vector (Clontech). Screening of a yeast 2-hybrid cDNA library derived from human fetal brain was performed as described (12).

RNA Interference and Generation of DsbA-L-Suppressed Cells. The sense and anti-sense sequences of siRNA were chemically synthesized and ligated into the pSIREN-RetroQ (BDKnoockout RNAi system; BD Biosciences). The sequence for siRNA and scrambled control are 5'-GCATGGAGCAACCAGAGAT-3' and 5'-GCCGTAGCACTGGAAGAAA-3', respectively. 3T3-L1 stable cell lines containing the DsbA-L siRNA construct or a scrambled control were generated by transfection of cells with the respected plasmids and selection with 3 μ g/ml of puromycin as described (42). Independent clones were selected by limited dilution.

Adenovirus Generation and Adenoviral Infection. Adenoviruses encoding GFP and mouse DsbA-L were generated by using the pAdEasy system as described (40). On differentiation day 9, 3T3-L1 CAR adipocytes were infected with adenoviruses at an multiplicity of infection of 30. The efficiency of adenovirus infection was assessed by fluorescent GFP expression with a fluorescence microscope 24 h after infection. To measure adiponectin in cell culture medium by Western blot, cells were incubated in serum-free DMEM containing 0.05% BSA for 24 h and the conditioned media were collected and concentrated with iCON concentrators (Pierce).

Size Exclusion Chromatography. A Superdex 200 10/30 column (GE Healthcare Bio-Sciences) was equilibrated with buffer containing 50 mM phosphate buffer,

pH 7.0, 150 mM NaCl, and 2 mM KCl and calibrated with standards: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; and ovalbumin, 44 kDa. Cell lysates were clarified by centrifugation at $12,000 \times g$ for 15 min at 4 °C. The clarified supernatant (≈ 0.5 mL) was applied to the column and eluted with the same buffer at a flow rate of 0.5 mL/min by FPLC. Fractions (0.1 mL of for cell lysates and 0.2 mL of for concentrated cell culture media) were collected and separated by SDS/PAGE, and the position of adiponectin in the elution profile was determined by Western blot by using an antiadiponectin or anti-HA tag antibody.

Pulse-Chase Experiments. Scramble or DsbA-L-suppressed 3T3-L1 cells were induced for differentiation for 8 days, starved in methionine- and cysteine-free DMEM for 1 h, and then replaced with the same fresh medium plus $50 \mu\text{Ci/ml}$ of [^{35}S]methionine and cysteine (Redivue Pro-mix L- ^{35}S , GE Healthcare) for 2 h. The labeling medium was then replaced with cold DMEM plus a 20-fold excess of methionine and cysteine for different time period. Both cell culture medium and cells were harvested for immunoprecipitation as we described (43).

DsbA-L Expression in Human Subjects. Thirty-five healthy premenopausal Chinese women (23 lean and 12 overweight/obese individuals, age: 43 ± 7 years) undergoing abdominal surgery for benign gynecological conditions, such as uterine fibroids or ovarian cysts, at the Department of Obstetrics and Gynecology, University of Hong Kong, Queen Mary Hospital, were recruited. All subjects were given informed consent, and the protocol was approved by the Ethics Committee of the University of Hong Kong. Preoperative serum, BMI, waist circumference,

waist-hip ratio, blood pressure, fat percentage, and glucose levels at 30-min intervals for 2 h during a 75 gram Oral Glucose Tolerance Test (OGTT) were determined. Serum adiponectin levels were measured as described (43, 44) (Table S2). During the operation, abdominal s.c. adipose tissues (≈ 2 cc each) were collected, snap-frozen, and stored at -70 °C before RNA extractions using the Qiagen mini-RNA purification kits. One microgram of total RNA from each sample was reverse-transcribed, and the relative mRNA abundance of DsbA-L was quantified by quantitative real-time PCR using the fluorescent TaqMan 5'-nuclease assay on an Applied Biosystems Prism 7000 sequence detection system. The TaqMan real-time PCR was performed by using 2' TaqMan Master Mix and $20\times$ assay-on-demand TaqMan primers and probes (Assay ID: CF02642588-81, Applied Biosystems). Analysis was performed with ABI Prism 7000 SDS Software.

Data Analysis. Statistical analysis of DsbA-L and adipokine mRNA levels in human was performed with the SPSS 11.5 statistical software package (SPSS). The data for mRNA, protein of human or mouse WAT and clinical characteristics were analyzed by using an unpaired *t* test, unless otherwise indicated. Data are presented as means \pm SEM, and *P* value of <0.05 was considered to be statistically significant.

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