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Modification of *N*-glycosylation modulates the secretion and lipolytic function of apoptosis inhibitor of macrophage (AIM) ☆

Mayumi Mori [1], Hiroki Kimura, Yoshihiro Iwamura, Satoko Arai, Toru Miyazaki *

Laboratory of Molecular Biomedicine for Pathogenesis, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

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ABSTRACT

The mouse macrophage-derived apoptosis inhibitor of macrophage (AIM), which is incorporated into adipocytes and induces lipolysis by suppressing fatty acid synthase (FAS) activity, possesses three potential *N*-glycosylation sites. Inactivation of *N*-glycosylation sites revealed that mouse AIM contains two *N*-glycans in the first and second scavenger receptor cysteine-rich domains, and that depletion of *N*-glycans decreased AIM secretion from producing cells. Interestingly, the lack of *N*-glycans increased AIM lipolytic activity through enhancing AIM incorporation into adipocytes. Although human AIM contains no *N*-glycan, attachment of *N*-glycans increased AIM secretion. Thus, the *N*-glycosylation plays important roles in the secretion and lipolytic function of AIM.

Structured summary of protein interactions: AIM physically interacts with FAS by anti tag coimmunoprecipitation (View interaction)

1. Introduction

The apoptosis inhibitor of macrophage (AIM, also called CD5L, Spα, or Api6) protein is a member of the scavenger receptor cysteine-rich superfamily (SRCR-SF) and was initially identified as an apoptosis inhibitor that supports the survival of macrophages against various apoptosis-inducing stimuli [1]. As a secreted molecule, AIM has been detected in human and mouse blood at varying levels [1–6]. AIM is produced by lipid-laden foam macrophages located within atherosclerotic plaques, and exacerbates the disease by supporting the survival of macrophages within lesions [7]. In addition, AIM is incorporated into mature adipocytes via CD36-mediated endocytosis where it suppresses the activity of cytosolic fatty acid synthase (FAS) by direct association resulting in lipolysis, the degradation of triacylglycerols into glycerol and free fatty acids (FFA) [8,9]. In obesity, the augmentation of blood AIM levels induces vigorous lipolysis in adipose tissues, increasing local extracellular fatty acid concentrations to a level sufficient for the stimulation of adipocyte-expressing toll-like receptor (TLR) 4, which triggers macrophage recruitment and chemokine production by adipocytes [10]. This response causes chronic, low-grade inflammation in adipose tissues, which is associated with insulin-resistance, and thus contributes to the development of multiple obesity-induced metabolic and cardiovascular diseases [8–11].

Both murine and human AIM (hAIM) possess several putative *N*-glycosylation sites (asparagine-X-threonine or serine; N-X-S/T, X ≠ P). However, the precise contribution of the *N*-glycans to the AIM function and/or other protein characteristics of AIM remain unsolved. Therefore, in this study, we investigated the effects of glycomodification on AIM function, focusing on its lipolytic effect, by generating variant AIM proteins with reduced or additional *N*-glycans from site-directed mutagenesis.

2. Materials and methods

2.1. Digestion of glycans

Deglycosylation was performed using Enzymatic Protein Deglycosylation Kit (Sigma, USA). Each type of AIM was produced in the culture supernatant of HEK293T cells and immune-precipitated with anti-HA antibody (Roche, Germany). The precipitates were

Abbreviations: AIM, apoptosis inhibitor of macrophage; SRCR, scavenger receptor cysteine-rich; FSP27, fat-specific protein 27; TLR, toll-like receptor; FAS, fatty acid synthase; QPCR, quantitative RT-PCR; IL-6, interleukin-6; Saa-3, serum amyloid A-3; DEX, dexamethasone; IBMX, isobutylmethylxanthine

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* Corresponding author. Address: 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Fax: +81 3 5841 1438.
E-mail address: tm@m.u-tokyo.ac.jp (T. Miyazaki).
[1] A JSPS Research Fellow.

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diluted in 50 mM phosphate buffer (pH 7.5) and incubated with PNGase F at 37 °C for 48 h. Endogenous AIM from mouse serum was immune-precipitated with anti-AIM antibody (rat, #12) and reacted with PNGase F in the presence of SDS and Triton X-100. For digestion of *O*-glycans, mixture of *endo*-*O*-glycosidase, α-2 (3,6,8,9)-neuraminidase, β-1,4-galactosidase, and β-*N*-acetylglucosaminidase as well as PNGase F was used.

2.2. Lectin blot

Five micrograms of purified AIM were used for SDS–PAGE and transferred on PVDF membrane. After blocking with 5% BSA-TBST, 20 μg/ml lectins were applied. Binding was detected with streptavidin-HRP. Normal mouse immunoglobulin G (Sigma) was used as a positive control.

2.3. FITC-labeling of AIM and uptake experiment

Purified AIM was labeled with FITC by using SureLINK™ Fluorescein (FITC) Labeling Kit (KPL, USA). Labeling efficiency was evaluated by measurement of absorbance at 280 nm (for protein concentration) and 490 nm (for fluorescein), confirming no difference between WT and ΔS1ΔS2 mAIM. At day 7 of adipocyte differentiation, 3T3-L1 cells were treated with various concentrations of FITC-AIM for 6 h. Cells were lysed in lysis buffer containing 1% NP-40 and 150 mM Tris–HCl (pH 7.5) after thoroughly washing with PBS. Uptake of FITC-AIM into 3T3-L1 adipocytes was quantified by measurement of 535 nm fluorescence. Values were normalized by protein concentration in the lysates.

2.4. Statistic

All statistical analyses were performed using the two-tailed Student's t-test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Descriptions for Reagents and antibodies, Procedures for Vector Construction, Purification of recombinant AIM, Lipolysis assay, Quantitative RT-PCR and primer sequences, and Co-immuno-precipitation assay, appear in Supplementary Materials and methods.

3. Results

3.1. Analysis of N-glycosylated sites in AIM

Since murine AIM (mAIM) has a larger molecular weight (~55 kDa) than predicted from its amino acid sequence (39 kDa), it is possible that mAIM is naturally glycosylated. The C57BL/6 (B6) strain mAIM amino acid sequence (NCBI: NP_033820) suggests that each of three SRCR domain possesses a potential *N*-glycosylation site; *i.e.* the asparagine (N) 99, N229, and N316 residues, respectively (Fig. 1A). Although we also found that the FVB/N and BALB/c mouse strains have a fourth *N*-glycosylation site at the N195 residue of AIM (GenBank: AAH94459 and AF018268.1, respectively), we used the B6-type AIM as wild-type (WT) in the present study. To verify the presence of *N*-glycans at each potential site, we generated three variant AIM recombinant proteins each containing a single *N*-glycosylation site in a different SRCR domain using combinational amino acid modifications of asparagine to glutamine at N99 (SRCR1), N229 (SRCR2), and N316 (SRCR3), and a fourth variant lacking an *N*-glycosylation site. Thus, variants ΔS2ΔS3, ΔS1ΔS3, ΔS1ΔS2, and ΔS1ΔS2ΔS3 harbor *N*-glycosylation sites in SRCR1, 2, 3, or none of the SRCR domains, respectively.

WT and variant mAIM proteins with an HA-tag at the C-terminal were produced in HEK293T cells, immunoprecipitated using an anti-HA antibody, and the precipitates were treated with the protein *N*-glycosidase F (PNGase F) under non-denaturing conditions. PNGase F treatment reduced the WT molecular weight to that of ΔS1ΔS2 and ΔS1ΔS2ΔS3, which were of identical size (Fig. 1B). ΔS2ΔS3 and ΔS1ΔS3 were intermediate in size between WT and ΔS1ΔS2ΔS3, which was reduced to that of ΔS1ΔS2ΔS3 after PNGase F treatment (Fig. 1B). These results suggest that WT mAIM possesses *N*-glycans at the SRCR1 and SRCR2 domains, and that the N316 in SRCR3 lacks an *N*-glycan.

We also attempted PNGase F treatment of endogenous mAIM after precipitating AIM from mouse serum using an anti-mAIM antibody. The molecular weight of endogenous AIM was identical to that of WT recombinant mAIM, but reduced to that of ΔS1ΔS2 after PNGase F treatment, as assessed by immunoblotting under reducing conditions (Fig. 1C), clearly suggesting that the endogenous blood mAIM possesses *N*-glycans like as seen in recombinant mAIM.

The hAIM has a smaller molecular weight compared with mAIM, although their predicted sizes from amino acid sequences are similar (~38 kDa). The hAIM amino acid sequence (NCBI: NP_005885) indicates the presence of a potential *N*-glycosylation site (asparagine-X-cysteine; NXC) [12,13] in the SRCR2 and SRCR3 domains. It was reported that the NXC motif might have the potential to attach *N*-glycans, though it is not a consensus site like mAIM N-X-T/S. However, PNGase F treatment did not reduce the molecular size of WT hAIM, suggesting no *N*-glycosylation at these NXC sites (Fig. 1D). This result is consistent with a previous observation by Gebe et al. suggesting that hAIM might not contain putative *N*-glycosylation [2].

3.2. Glycomodification profiling in AIM

To determine the patterns of carbohydrate chains in WT and variant AIM proteins, we employed five different lectins which recognize variable motifs of the sugar attachment. As shown in Fig. 1E, concanavalin A (Con A), which recognizes all types of branched *N*-glycans, recognized WT, ΔS1, and ΔS2, but not ΔS1ΔS2 mAIM. The *Sambucus nigra* agglutinin (SNA), but not the *Maackia amurensis* agglutinin (MAA), reacted with WT mAIM, suggesting that the two mAIM *N*-glycans possess α2,6- but not α2,3-linked sialic acids. Although the *Ulex europaeus* agglutinin (UEA-I) detected no terminal fucose in WT mAIM, the *Erythrina cristagalli* agglutinin (ECA) blot revealed the presence of terminal *N*-acetylgalactosamine in the second mAIM *N*-glycan at N229 (SRCR2). This suggests that the *N*-glycan at N99 (SRCR1) possesses only α2,6-sialylated terminals, and the one at N299 (SRCR2) possesses both α2,6-sialylated and non-sialylated terminals.

Since any mutation in the amino acid sequence may affect the receptiveness of *O*-linked glycosylation, we also evaluated the state of *O*-glycosylation in WT and variant AIM proteins by treating them with three different exoglycosidases and one endoglycosidase. No *O*-glycan was detected in either mAIM or hAIM (Supplementary Fig. 1A). According to the online database (NetOGlyc 3.1 Server, http://www.cbs.dtu.dk/services/NetOGlyc/), there are four potential *O*-glycosylation sites located at serine (S)123, S129, S130, and S132 within the hinge-region linking SRCR1 and SRCR2 domains of hAIM. However, their potentiality scores are only around 0.38, which is below the threshold of 0.50. To further test the presence of *O*-glycosylation in hAIM, we generated a variant hAIM protein harboring a substitution of alanine for serine at all of these potential sites. The size of this variant hAIM (Δ4O) was similar to that of WT hAIM protein, implying no prominent *O*-glycosylation in hAIM (Supplementary Fig. 1B).

3.3. N-glycosylation increases AIM secretion

We next investigated the influence of different glycomodifications on the functional characteristics of AIM. We first tested the secretion of variant AIM proteins (ΔS1 and ΔS2, possessing a single *N*-glycan at SRCR2 and SRCR1, respectively, and ΔS1ΔS2 with