Identification of ISG12b as a Putative Interferon-inducible Adipocytokine which is Highly Expressed in White Adipose Tissue

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Aim: A number of adipocytokines have been suggested to be involved in the disruption of glucose metabolism, and also in the development of various diabetic complications. We attempted to identify and analyze additional adipocytokines, to better understanding the roles of adipocytes and adipocytokines.

Methods: An oligo-capping signal sequence trap, developed in our laboratory for screening the cDNAs of secretory proteins, was used to screen cDNAs expressed in mouse white adipose tissue. Profiles of the genes identified in mice and cultured cells were further investigated by northern blotting and luciferase assay.

Results: A cDNA fragment of interferon-stimulated gene 12b (ISG12b) was obtained in the search. A northern blot analysis revealed ISG12b to be highly expressed in white adipose tissue. Interferon α (IFNα) was shown to induce ISG12b expression in the adipose tissue of BL6 mice in vivo, and also in a 3T3-L1 preadipocyte cell line in vitro. The level of ISG12b was higher in mature adipocytes than in preadipocytes. A promoter analysis demonstrated that the 369bp upstream from the transcription initiation site of ISG12b mRNA contain strong promoter activity, and the interferon-stimulated response elements (ISREs) were not present within the 5593bp upstream region.

Conclusion: ISG12b is an additional candidate for a adipocytokine induced to express in adipose tissue by interferon.


Key words: Interferon-stimulated gene, Adipose tissue, Adipocytokine, Interferon

Introduction

Adipose tissue is a major target in the study of the mechanism behind the development of resistance to insulin, obesity, and type 2 diabetes. For the past decade, special attention has focused on a number of secretory proteins from the adipocytes called adipocytokines. These include adiponectin, leptin, resistin, interleukin-6, and tumor necrosis factor-α (TNFα). Such adipocytokines have been suggested to be involved in the disruption of glucose metabolism, and also in the development of various diabetic complications.

Adiponectin is exclusively expressed by mature adipocytes, with an increase in expression and secretion during the process of adipocyte differentiation, and levels of adiponectin are lower in visceral fat than subcutaneous fat¹, ². It has been reported that increased plasma concentrations of adiponectin are associated with a reduced risk of type 2 diabetes³. In addition, in mice lacking adiponectin, injury-induced neointimal hyperplasia was shown to develop even under normal conditions of glucose and lipid metabolism, suggesting adiponectin to be directly involved in anti-
arteriosclerosis and anti-inflammation\textsuperscript{4}).

Leptin is secreted from mature adipocytes and acts through the hypothalamus to control appetite and body weight\textsuperscript{5, 6}. A deficiency of leptin has been shown to be important in certain cases of lipodystrophy, a rare disease characterized by a paucity of adipose tissue accompanied by severe resistance to insulin, thus leading to the development of diabetes mellitus, fatty liver, and vascular diseases. Treatment with leptin replacement therapy has been shown to improve glycemic control and other metabolic abnormalities in these patients\textsuperscript{6, 7}.

The administration of resistin to mice has been shown to impair glucose tolerance and insulin action. Plasma concentrations of resistin were higher in genetic and diet-induced obese mice with insulin resistance. Injection of a neutralizing antibody against resistin increased insulin sensitivity in obese mice\textsuperscript{7, 8, 9}. These results suggest resistin to be an adipose-tissue-derived factor causing insulin resistance in those with obesity. It has also been shown that the insulin-sensitizing effect of glitazone is partly attributable to the inhibition of resistin expression\textsuperscript{9, 10}.

Interleukin-6 is mainly produced in adipose tissue (10-30%), and its serum concentration has been shown to be increased in obese patients\textsuperscript{11}. In addition, interleukin-6 has been reported to induce insulin resistance based on measurements of the uptake of glucose by adipocytes with the addition of insulin in vitro\textsuperscript{12}.

Production of TNF\(\alpha\) also occurs in white adipose tissue, and is markedly up-regulated in obese individuals\textsuperscript{13}. It has been suggested that inhibition of TNF\(\alpha\) may block the development of insulin resistance associated with diet-induced obesity. In addition, this cytokine has a variety of functions in the development of inflammation and atherosclerosis. TNF\(\alpha\) is a potent inhibitor of adiponectin synthesis, and an increase in the secretion of TNF\(\alpha\) from accumulated visceral fat may be one cause of the reduction in the amount of adiponectin in individuals with visceral obesity.

As there may be more adipocytokines involved in the control of glucose metabolism, we attempted to screen for unidentified secretory factors expressed in adipocytes using an oligo-capping signal sequence trap (oligo-cap SST). The oligo-cap SST was developed in our laboratory for screening the cDNAs of secretory proteins. First, cDNA libraries specifically containing the sequences of the 5' region of mRNAs are constructed by utilizing a combination of oligo-capping and the polymerase-chain reaction (PCR). The SST method is the applied, which is an efficient way of screening for cDNA fragments containing signal sequences. We cloned the cDNA of interferon-stimulated gene 12b (ISG12b) through such a screening. ISG12b was originally identified in human breast carcinoma cells\textsuperscript{14}, and its expression has been shown to be induced by interferon\textsuperscript{15, 16}, which is a cytokine involved in the control of various inflammatory and anti-inflammatory responses. Investigation of the expression of ISG12b in adipose tissue suggests that ISG12b is an interferon-regulated adipocytokine involved in inflammation, and possibly also in the development of metabolic disorders and various vascular complications.

**Materials and Methods**

**Animal Experiments**

All mice were kept under specific pathogen-free conditions with free access to a standard commercial diet. Male 8-wk-old BL6 mice were injected with 10\(^4\) U of recombinant mouse interferon alpha A (PBL Biomedical Laboratories) or phosphate-buffered saline (PBS) per day, intraperitoneally, and sacrificed one week after the first injection. To induce diabetes, the mice were treated with streptozotocin (STZ) (100 mg STZ/kg body weight per day). Plasma glucose concentrations were measured to confirm the development of diabetes after a two-week period. All the animal husbandry and animal experiments were consistent with the University of Tsukuba’s Regulation of Animal Experiments and were approved by the Animal Experiment Committee, University of Tsukuba.

**Cell Culture**

3T3-L1 preadipocytes were maintained in high-glucose (25 mM) DMEM with 5% calf serum. The medium was changed every other day, and the cells were split and passed before they reached confluence. Twenty-four hours after the 3T3-L1 cells were plated on a dish, recombinant mouse interferon alpha A (PBL Biomedical Laboratories) (100 U/mL or 1,000 U/mL) was added to the medium. For the induction of differentiation, after reaching confluence, 3T3-L1 cells were incubated in an induction medium of high-glucose DMEM containing 5% fetal bovine serum, 0.25 \(\mu\)M dexamethasone, 200 \(\mu\)M IBMX, and 5 \(\mu\)g/mL insulin, for forty-eight hours. At postinduction day (PID) 3, the medium was changed to a growth medium containing high-glucose DMEM and 5% fetal bovine serum. The culture medium was routinely changed every other day. PID 0 refers to just before addition of the induction medium. After the induction of differentiation, the 3T3-L1 cells were cultured until PID 7.

**Preparation of Adipocytes**

Mature adipocytes were prepared according to the method of Rodbell as follows: hypodermal fatty tissue
of 8-week-old BL6 mice was minced into small pieces with a pair of sharp scissors and then digested in a 0.2% collagenase solution at 37°C for 30 min. The adipocytes were suspended in the solution and the resulting cell suspension was then filtered through mesh. After centrifugation, the mature adipocytes that floated to the top and the preadipocytes that precipitated to the bottom were collected separately.

**Generation of ISG12b genes**

The 5' flanking sequence of the mouse ISG12b1 gene, located on Chromosome 12, was identified by GenBank (NT_039551.6|Ms12_39591_36:6138344-61386852). The ISG12b1 probe for the northern blot analysis comprised the 682bp fragment of the ISG12b1 coding region (NT_039551.6|Ms12_39591_36:6138197-61387516). The ISG12b1 promoter was amplified from the BL6 genomic DNA by PCR.

**Northern Blot Analysis**

Total RNA from 3T3-L1 cells and mouse tissue was isolated using Trizol (Invitrogen), and were fractionated and analyzed by northern hybridization. Ten micrograms of total RNA was applied to each lane in a 1% agarose gel with formaldehyde, and transferred to Hybond N+ (Amersham) membranes. These membranes were prehybridized at 65°C for 1 h. The ISG12b1 probe was labeled using a multiprime DNA labeling system (Amersham) with (α-32P) dCTP. The probes were hybridized to the membranes in the hybridization solution at 65°C for 1.5 h. The membranes were washed three times (15 min for each wash) in 0.1%SDS 1× SSC at 65°C. The blots were analyzed by a phosphoimager.

**Generation of ISG12b Promoter-luciferase Reporter genes**

The promoterless luciferase reporter gene plasmid pGL3basic (Promega) was used to construct ISG12b1 promoter reporter plasmids. PCR was used to amplify the DNA fragments from the BL6 mouse genome for the luciferase assay. The −2170bp fragment was amplified with primers 5'-CCGGTACCGAATTCAAGGTAAAC-3' (forward) and 5'-GGACCGGTCTCTAGAGGAC-TACATCCAGAAC-3' (reverse), for the −3160/−2170bp fragment, 5'-CCGGTACCGGACTGAGG-GGCTCAGTG-3' (forward) and 5'-GGACCGGT-TGCTCTGAGACTCCTCC-3' (reverse), and for the −5593/−3160bp fragment, 5'-CCGGTACCATCCTTGTAGAGGG-3' (forward) and 5'-GGACGGTG ACTGAGCCTTACGTCC-3' (reverse). The −871bp fragment was prepared by digestion with Pst I from the −2170/−29bp fragment. The −2170/−617bp fragment was prepared by digestion with Xho I from the −2170/−29bp fragment.

**Transfection and Luciferase Assay**

3T3-L1 cells were plated at a density of 10,000 cells/well on 24-well plates and cultured overnight. The pGL3 basic vectors or pGL3 promoter vectors and renilla luciferase reporter vectors were co-transfected into 3T3-L1 cells with FuGENE 6 transfection reagent (Roche) following the manufacturer’s instructions. Recombinant mouse interferon alpha A (PBL Biomedical Laboratories) was added to the medium 12 hours after transfection, at a concentration of 100 U/mL. The cells were cultured for 36 hours and harvested, and relative activity was evaluated using the Promega luciferase assay kit (Promega).

**Results**

**Identification of ISG12b by Oligo-cap SST Screening**

Screening was performed using the mRNA extracted from epididymal fat pad obtained from BL6 mice by the oligo-cap SST method. Of the 300 clones obtained from the initial screening, 23 turned out to be the ISG12b gene.

The cDNA of ISG12b was originally cloned as a gene expressed in a human breast epithelial cell line, MCF7. The expression of ISG12b has been shown to be induced by interferon-α (IFNα) in a number of cell lines including HeLa, MCF7, and HFL1. However, the function of ISG12b has not been sufficiently elucidated.

**ISG12b is Highly Expressed in White Adipose Tissue**

We performed a northern blot analysis to investigate the expression of ISG12b in various mouse tissues (Fig. 1). ISG12b was highly expressed in white adipose tissue, but showed minimal expression in brown adipose tissue and kidney. ISG12b was not detectable in brain, lung, heart, liver, spleen, intestine, and muscle tissue specimens. The size of the ISG12b mRNA
was approximately 0.65 kb, which is consistent with the length of a cDNA reported by Parker \textit{et al.}\textsuperscript{16}. We concluded that white adipose tissue is the major tissue where ISG12b is expressed \textit{in vivo}.

**Interferon-\(\alpha\) Induces the Expression of ISG12b in Mouse 3T3-L1 Cells**

To examine whether interferon induces the expression of ISG12b in adipocyte-derived cells \textit{in vitro}, we used 3T3-L1 cells. We added IFN\(\alpha\) to the medium of the 3T3-L1 cells to determine if IFN\(\alpha\) increases ISG12b expression. ISG12b was shown to be highly expressed in the 3T3-L1 cells treated with IFN\(\alpha\) (Fig. 2A).

**Interferon-\(\alpha\) Induces the Expression of ISG12b in White Adipose Tissue \textit{in vivo}**

We next tested whether IFN\(\alpha\) can induce the expression of ISG12b in adipose tissue \textit{in vivo}. IFN\(\alpha\) (10\(^4\) U) was injected every day intraperitoneally into BL6 mice for a 1-week period, and the expression of ISG12b in the white adipose tissue was examined. Higher levels of ISG12b in white adipose tissue were found in the mice injected with IFN\(\alpha\), than in the mice injected with saline (Fig. 2B).

**Promoter Analysis of ISG12b**

In order to investigate the mechanism by which ISG12b is expressed in the adipocytes, we searched for the ISG12b promoter region. The putative promoter region of ISG12b was amplified from the BL6 mouse genome using the PCR method. A fragment containing the 2170 bp upstream of the putative transcriptional start site of the ISG12b gene was inserted into the pGL3 basic vector. Reporter plasmids containing serial deletions from the 5’ side of this fragment were constructed and also tested for activity. Plasmids were transfected into the 3T3-L1 cells, and then the LUC assay was performed. The 391 bp upstream of the putative transcriptional start site were shown to contain sufficient promoter activity in the 3T3-L1 cells (Fig. 3A).

To search for the interferon-stimulated response elements (ISREs), the ISG12b gene promoter region was divided into four fragments, and each tested for a response to IFN (Fig. 3B). However, the presence of ISREs was not demonstrated within any of these fragments including the fragment containing a putative ISRE sequence predicted by Parker \textit{et al.}\textsuperscript{16}.

**Differentiation of Adipocytes and the Expression of ISG12b**

Next, white adipose tissue of BL6 mice was separated into mature and immature adipocytes, and the expression of ISG12b was examined. As expected, strong expression of leptin was seen only in the mature adipocytes. Interestingly, ISG12b was also found to be strongly expressed in mature adipocytes compared to immature adipocytes (Fig. 4A).

Next, to investigate a possible involvement of ISG12b in the differentiation of adipocytes, expression of ISG12b was examined in the 3T3-L1 cells induced to differentiate. The expression of ISG12b was decreased after the differentiation was induced on PID 2, and then gradually increased as the differentiation proceeded (Fig. 4B).
The Expression of ISG12 in ob/ob Mice and Diabetic Mice

The expression of ISG12b in white adipose tissue was examined in BL6 mice, STZ mice, and ob/ob mice. Levels were lower in the ob/ob mice than in the other mice (Fig. 4C).

Discussion

In this study, screening for secretory proteins expressed in white adipose tissue was performed using an oligo-cap signal sequence trap developed in our laboratory. A cDNA fragment of interferon-stimulated gene 12b (ISG12b) was obtained by the screening.

ISG12b was first reported as an oestrogen-induced gene (originally designated p27) in a human breast epithelial cell line, MCF7. The level of ISG12b was increased in breast cancer, thus suggesting the possible involvement of this gene in carcinogenesis. The expression of ISG12 has been shown to be induced by IFN-α in the human cell lines HeLa, MCF7, and HFL1. However, the function of ISG12b still remains to be elucidated.

A northern blot analysis demonstrated ISG12b to be highly expressed in white adipose tissue, but not in brain, lung, heart, liver, spleen, intestine, or muscle tissue. Minimal expression was seen in both kidney and brown adipose tissue. Interferon α (IFNα) has been shown to induce the expression of ISG12b in the 3T3-L1 preadipocyte cell line in vitro, and also in the

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**Fig. 3.** Promoter analysis of ISG12b gene expression.

A) Examination of the promoter region of ISG12b by LUC assay in 3T3-L1 cells. The region -2170bp upstream of translation start site of the ISG12b gene was inserted into pGL3 basic vector. Mutants containing the region indicated (-691bp; 691bp upstream of translation start site, -396; 396bp upstream of translation start site) were made using deleted fragments of the promoter region from the 5′ side. The pGL3 basic vectors were used as a control.

B) Examination of the interferon-stimulated response element (ISRE) by LUC assay in 3T3-L1 cells. The promoter region of ISG12b was divided into four fragments as indicated (-5593 to -3160bp, -3160 to -2170bp; -2170 to -617bp, -617 to -29bp). The effect of IFNα on each fragment was examined. The pGL3 promoter vectors were used as a control.

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**Fig. 4.** Expression of ISG12b in adipocytes in different stages of differentiation, and in model mice.

A) Expression of ISG12b in mature and immature adipocytes as determined by northern blotting. The expression of leptin was verified as a control.

B) Expression of ISG12b in differentiating 3T3-L1 cells as determined by northern blotting. Postinduction day (PID) 0 refers to just before differentiation was induced. 3T3-L1 cells were cultured until PID 7. EF1α was used as a control for northern blotting.

C) The expression of ISG12b in BL6 mice (n = 2), STZ mice (n = 2), and ob/ob mice (n = 2).
white adipose tissue of BL6 mice in vivo. Furthermore, levels of ISG12b have been shown to be higher in mature adipocytes than in preadipocytes. In addition, in 3T3-L1 cells which were stimulated to differentiate, the expression of ISG12b has been shown to increase as the differentiation proceeds. These findings suggest that ISG12b is involved in the maturation and differentiation of adipocytes. Interestingly, the expression of ISG12b was relatively decreased in the white adipose tissue of ob/ob mice. Ob/ob mice are deficient in normal leptin activity, which leads to obesity and diabetes. Leptin has been reported to affect the expression of various genes in white adipose tissue and may be involved in the development of metabolic disorders. Although we do not know the role of ISG12b in white adipose tissue, it would be interesting to investigate whether ISG12b is involved in the regulation of metabolism, possibly in combination with leptin action.

A promoter analysis has demonstrated the 369bp upstream of the transcription initiation site of ISG12b mRNA to contain strong promoter activity, though no ISREs were identified within 5593bp upstream. The mechanisms by which interferon induces the expression of ISG12b remain to be elucidated.

Our findings showed that ISG12b is an additional candidate for an adipocytokine induced to express in adipose tissue by IFNα, a cytokine known to be involved in immunological and inflammatory processes. Inflammatory and anti-inflammatory reactions are thought to play important roles in the development of various vascular diseases including atherosclerosis and diabetic complications. Further analysis to investigate whether and if so how ISG12b is involved in these processes, and also in the differentiation of adipocyte will be necessary.

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