RGS2 Promotes Adipocyte Differentiation in the Presence of Ligand for PPARγ*

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SUMMARY

The events at the earliest stage of adipocyte differentiation are yet to be fully elucidated. Previously, we cloned the genes which are induced at the beginning of the differentiation of mouse 3T3-L1 preadipocyte cells. We found that the gene expression of regulators of G protein signaling 2 (RGS2) rapidly increased after the addition of inducers, and decreased at 3-12 hrs. The expression pattern of RGS2 mRNAs differed among growth-arrested and proliferating 3T3-L1 cells and NIH-3T3 cells, indicating a specificity for adipogenesis. Here we have reported that the ectopic expression of RGS2 using a retroviral system in mouse NIH-3T3 cells promotes adipogenesis only in the presence of BRL49653 which is a ligand for the peroxisome proliferator-activated receptor γ (PPARγ). These results strongly suggest that RGS2 play a crucial role in the program of adipocyte differentiation and may contribute to the function of PPARγ.
INTRODUCTION

Adipose tissue is the main organ for energy storage, and secretes many kinds of cytokines such as leptin and resistin (1-3). Too much fat, however, causes severe diseases including diabetes and heart failure (4). Therefore, greater insight into the molecular basis of obesity is required (5).

The differentiation of adipocytes has been relatively well characterized and several transcription factors identified as master regulators for the process (6, 7). Peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)) is a specific transcription factor active during adipogenesis. Indeed, a mouse fibroblast cell line, NIH-3T3, stably infected with PPAR\( \gamma \) expression vector differentiated into adipocytes (6-8). The CCAAT/enhancer-binding protein (C/EBP) family also are identified as master regulators, and the ectopic expression of C/EBP\( \alpha \) or C/EBP\( \beta \) induces PPAR\( \gamma \) expression and adipogenesis (9). C/EBP\( \beta \) is known to be an activator for C/EBP\( \alpha \), and the expression of C/EBP\( \beta \) and C/EBP\( \delta \) is preceded by that of C/EBP\( \alpha \) (6). Interestingly, in double knockout mice for C/EBP\( \beta \) and CEBP\( \delta \), adipogenesis was inhibited and the amount of stored fat was reduced, although PPAR\( \gamma \) and C/EBP\( \alpha \) were expressed normally (10). These results have indicated that the differentiation is much more complex than previously thought and may have multiple routes.

Whereas the middle and late stages of adipogenesis are well-studied, little is known about the earliest step of adipocyte differentiation. Mouse 3T3-L1 preadipocyte cells, which are fibroblastic cells of adipocyte lineage, are widely used for studies on adipocyte differentiation (11, 12). Using this cell line, the levels of over 300 cellular proteins were shown to change during the initiation of differentiation, and in many cases, the level of mRNA changed also (13). The proteins differentially expressed during adipocyte development were also summarized, and include proteins associated with hormone action and signaling, lipogenesis and lipolysis, and cytoskeletal and extracellular structures as well as secreted proteins (11). Thus, numerous uncharacterized cDNAs which are
differentially expressed during the conversion of adipocytes have been cloned (14-16). However, the expression of these cDNAs/proteins alone cannot explain the triggering of adipocyte differentiation.

Previously we isolated the regulator of G protein signaling 2 (RGS2) as the product of a gene which was strongly expressed at the beginning of adipocyte differentiation (17). RGS proteins are known to be negative regulators of G protein-dependent signaling, stimulating hydrolysis of the GTP bound to the activated Gα subunits (18). Although the RGS gene family consists of more than twenty members, not one has yet been reported to be directly involved in adipocyte differentiation. In our previous report, RGS2 was induced only in growth-arrested cells not in proliferating cells, indicating that this elevation is specific to the differentiation (17). These findings prompted us to further characterize the function of RGS2 during adipocyte differentiation. Here we report that the ectopic expression of RGS2 stimulates the adipogenesis of a fibroblastic cell line, mouse NIH-3T3, only in the presence of the ligand for PPARγ, and RGS2 seems to have a crucial role in adipogenesis.
Plasmid Construction-A full-length cDNA encoding mouse RGS2 was prepared by polymerase chain reaction (PCR) using cDNA made from total RNA after 3 hrs induction as a template. The mouse cDNAs encoding lipoprotein lipase (LPL) and aP2 were isolated from cDNA template made from total RNA 8 days induction by PCR, and subcloned into pBluescript KS(+) for expression of RGS2 by a retrovirus system, mouse RGS2 cDNA was subcloned into pDON-AI (Takara Biomedicals, Kusatsu, Japan) using SalI site.

Cell Culture-NIH 3T3 (clone 5611, JCRB 0615) fibroblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. For the differentiation experiment, the medium was changed to DMEM supplemented with 10% fetal bovine serum (FBS), 10 µg/ml of insulin (Ins), 0.5 mM 3-isobutyl-1-methylxanthine (Mix), and 1 µM dexamethazone (Dex) at 2-days post-confluence (13, 17). BRL49653 (5-(4-[2-(N-methyl-N-(2-pyridyl)amino)ethoxy]benzyl)thiazolidine-2,4-dione maleic acid salt, a gift from Smithkline Beecham Pharmaceuticals) was also added at a final concentration of 0.5 µM. PT67 packaging cell line (Clontech Lab. Inc., Palo Alto, CA) was maintained in DMEM containing 10% FBS. The 3T3-L1 (ATCC CL173) preadipocytes were maintained in DMEM containing 10% calf serum, and the differentiation was induced as described above except that BRL49653 was not added (17).

Stable Transformant Expressing RGS2-A stable transformant expressing RGS2 was established using Retro-X System (Clontech Lab. Inc.,) according to the manufacturer's instructions, except that pDON-AI was used as a retroviral vector. In brief, pDON-AI-RGS2 or pDON-AI was transfected into PT67 cells according to the calcium phosphate co-precipitation techniques described by Chen and Okayama (19). The viruses transiently expressed were collected, and
used to infect NIH-3T3 cells. After 14 days cultivation in the presence of 400 µg/ml of G418, a single clone was isolated.

**RNA Isolation and Northern Blot Analysis** - The cells were cultured and then harvested. Total RNA was extracted by using TRIzol (Gibco BRL Life Technologies, Inc., Grand Island, NY). For Northern blot analysis, 25 µg of total RNA was electrophoresed on a 1.0% agarose gel containing 2% formaldehyde, and then transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech Ltd.). The filter was hybridized with each probe labeled with [α-32P]-dCTP using a random labeling kit (Takara Biomedicalals, Kusatsu, Japan).
RESULTS

The Establishment of RGS2-Expressing NIH-3T3 Cells—Using the retroviral system, a stable transformant expressing RGS2 ectopically was isolated. In Fig. 1, the results of Northern blot analysis are shown. Using the plasmid pDON-AI as a probe, expression derived from empty and RGS2 vectors was observed at 4.6Kb and 4.0Kb, respectively (Fig.1A). When RGS2 was used as a probe, in addition to the endogenous expression of RGS2 (2.0Kb), ectopic expression derived from pDON-AI-RGS2 (4.6Kb) was detected (Fig. 1B). The approximate sizes of mRNAs derived from empty vector and pDON-AI-RGS2 were consistent with the calculated value.

----- Fig. 1. ----- 

The Conversion of RGS2-Expressing Cells to Adipocytes—Next we performed differentiation experiments. After 2 days, confluent stable transformants were given differentiation medium containing Mix, Dex, Ins and FBS. Under these conditions, neither RGS2-expressing nor control cells differentiated into adipocytes (data not shown). However, when BRL49653, which is a ligand for PPARγ, was added to the medium, the RGS2-expressing fibroblastic cells started to differentiate in a week (Fig. 2). Droplets of fat in the cells were also detected by Oil Red O staining, while the cells integrated with empty vector remained fibroblasts and did not store the oil.

----- Fig. 2. ----- 

The Expression of Several Markers for Adipogenesis in RGS2-Expressing Cells—Northern blot analysis was performed using several adipogenic markers as probes (Fig. 3). The expression of both PPARγ and sterol regulatory element binding protein 1 (SREBP-1), two of the master regulators of adipogenesis, was
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elevated during the differentiation. The levels of LPL and aP2 mRNAs were also found to elevate after 10 days of incubation. On the other hand, the expression levels of the C/EBP family were the same between RGS-expressing and control cells. Thus, the differentiation of RGS2-expressing fibroblasts to adipocytes requires the PPARγ ligand.

----- Fig. 3. -----
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DISCUSSION

During the adipocyte differentiation, C/EBPβ and C/EBPδ were expressed at a relatively early stage and induced the PPARγ as well as the C/EBPα gene (8, 9, 13, 20). For the induction, cAMP and glucocorticoid are known to be primary inducers (20). However, the earliest step in the differentiation of adipocytes is still not clear, though the activation of CREB is necessary and sufficient to induce adipogenesis (21). PPARγ expression is sufficient for the adipocyte differentiation, whereas double knockout mice for C/EBPβ and C/EBPδ expressed PPARγ, but did not store fat (10). Thus, it seems that the process for the differentiation to adipocyte is quite complex.

We previously isolated the genes induced at the earliest stage of the differentiation (17). RGS2 was particularly strongly expressed after the addition of inducers. This induction was stage-specific; occurring in the confluent cells, but not the proliferating cells, indicating that the expression of RGS2 is closely related to the differentiation. More than twenty genes are known to be part of the RGS family (18, 22, 23). The expression levels of RGS2, RGS5, and RGS16 were determined by PCR. Although these experiments were semi-quantitative, induction of RGS2 expression was observed. On the other hand, the levels of RGS5 and RGS16 were not changed (data not shown). Moreover, the localization of these RGSs was determined by transfection of GFP-fused RGS constructs. RGS2 was partially localized to the nucleus, while RGS5 and RGS16 were found in the cytosolic compartment (data not shown). The localization of RGS2 to the nucleus was reported quite recently (24, 25). These results strongly indicate that RGS2 has an important and specific function during adipocyte differentiation.

To further analyze the role of RGS2, we introduced a RGS2 expression vector into mouse NIH-3T3 cells, fibroblastic cells which do not differentiate into adipocytes in the presence of inducers. The resultant stable transformant did not differentiate on addition of a mixture of Mix, Dex, Ins and FBS. However, on
addition of this mixture plus BRL49653, which is a thiazolidine derivative and a ligand for PPARγ, RGS2-expressing stable transformants started to differentiate into adipocytes and stored oil droplets. The expression of adipogenic markers such as PPARγ, SREBP-1, LPL and aP2, was also observed. However, the levels of the three C/EBP members (C/EBPα, β, and δ) were unchanged. These results strongly suggest that the overexpression of RGS2 is sufficient for the differentiation to adipocyte through the PPARγ route, but has no effect on the expression of C/EBP.

What is the role of BRL49653? One possibility is that the low-level expression of PPARγ in NIH-3T3 cells activated genes in the presence of the ligand, and this effect was enhanced by the overexpression of RGS2. Alternatively, BRL49653 was utilized as a ligand for the unidentified receptor. It is clear that RGS2 has a critical role in the differentiation, although the detail is still unknown.

RGS2 was reported recently to reduce T cell proliferation as well as to control synaptic development (26). These results also indicated a role for RGS2 in the control of differentiation and proliferation. The real function of RGS2 during adipogenesis is still unknown, but the signals downstream of RGS2 are now under investigation at both the gene and protein expression level.
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Footnotes

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¹The abbreviations used are: RGS, regulator of G protein signaling; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT/enhancer binding protein; Dex, dexamethazone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Ins, insulin; LPL, lipoprotein lipase; PCR, polymerase chain reaction; SREBP-1, sterol regulatory element binding protein 1.
Figure Legends

**Fig. 1. Ectopic expression of RGS2 in NIH-3T3 stable transformants.**
Northern blot analyses were performed using stable transformants containing the integrating empty vector (pDON-AI) or RGS2 (pDON-AI-RGS2) in NIH-3T3 cells. The blots were hybridized with pDON-AI vector (A), and RGS2 (B), respectively. The arrows and arrowhead indicate the exogenous expression derived from empty vector or RGS2, and endogenous expression of RGS2, respectively.

**Fig. 2. Differentiation of RGS2-expressing stable transformants to adipocytes in the presence of PPARγ ligand, BRL49653.**
The RGS2-expressing stable transformant or control (empty vector-integrated) stable transformant was treated with differentiation medium containing Mix, Dex, Ins and FBS in the presence of BRL49653. The cells after ten days treatment were stained with Oil Red O.

**Fig. 3. Northern blot analyses of various gene mRNAs during adipocyte conversion of RGS2-expressing stable transformants.**
The RGS2-expressing stable transformant or control (empty vector-integrated) stable transformant was treated with differentiation medium containing Mix, Dex, Ins and FBS in the presence of BRL49653. The total RNA from cells after incubation were isolated for Northern blot analyses. The arrow and arrowhead in the C/EBPα panel show specific and nonspecific bands, respectively, although the expression of C/EBPα is almost undetectable.
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Fig. 2. Nishizuka et al.,
Fig. 3. Nishizuka et al.,