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Journal of Cellular Biochemistry 105:922–930 (2008)
Journal of Cellular Biochemistry

Characterization of the Transcriptional Regulation of the Regulator of G Protein Signaling 2 (RGS2) Gene During 3T3-L1 Preadipocyte Differentiation

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ABSTRACT
Adipocyte differentiation is a complex process involving several signaling pathways. Molecular mechanisms regulating the very early stage of adipocyte differentiation is not fully appreciated yet. Several inducible genes at the early stage of preadipocyte differentiation have been identified, including the regulator of G protein signaling 2 (RGS2), a member of the RGS protein superfamily. This study aimed to clarify the precise induction profile of RGS2 and to determine the essential transcription element(s) regulating RGS2 expression in differentiating 3T3-L1 preadipocytes. RGS2 mRNA expression was elevated immediately at 1 h after differentiation initiation and it remained high until the late stage of differentiation. The putative promoter sequence (~3,000 bp) of the mouse RGS2 gene was isolated and the RGS2 promoter activity was significantly upregulated 3 h after inducing differentiation. The primary signaling pathway leading to RGS2 transcriptional activation appeared to be cAMP-dependent. Sequential deletion and site-directed mutagenesis strategies demonstrate that the RGS2 promoter sequence truncated down to 78 bp in size retained full inducibility by the differentiation stimuli. Mutation of a Sp1 site within the 78 bp region significantly blocked promoter activity. In addition, high expression of Sp1 transcription factor was noted prior to and paralleling the differentiation process. Taken together, our data suggest that RGS2 transcription is immediately induced via a cAMP-dependent pathway after initiation of 3T3-L1 differentiation and the RGS2 mRNA level remains consistently high throughout the differentiation progression. A Sp1 site within RGS2 promoter appeared to be a crucial response element to regulate RGS2 transcription. J. Cell. Biochem. 105: 922–930, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: RGS2; 3T3-L1 PREADIPOCYTES; ADIPOCYTE DIFFERENTIATION

Adipose tissue is a reservoir of energy storage and produces a variety of molecules to modulate diverse physiological events. Adipocytes exert their physiological significance by regulating the functions of various tissues, including vasculature, liver, muscle, brain, reproductive tract and immune system, and thus they are involved in the development of several diseases, including cardiovascular disease, obesity, diabetes, cancer, and infectious disease [Kahn et al., 2006; Pi-Sunyer, 2006; Van Gaal et al., 2006]. Adipocyte differentiation is obviously an important step in the determination of adipose tissue composition and its associated functions [Rosen and Spiegelman, 2006].

During adipocyte differentiation, several transcription factors are induced and of these, the transcription factors CCAAT/enhancer-binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) families have been demonstrated to play crucial roles during adipocyte maturation [Lane et al., 1999; Lehrke and Lazar, 2005; Rosen, 2005]. However, knowledge is still lacking on the regulation of the very early stage of how differentiation initiation occurs at the molecular level. From a microarray analysis of differentiating 3T3-L1 preadipocytes in the early stage, one RGS (regulator of G protein signaling) protein, RGS2, had been previously shown to be dramatically induced after induction of differentiation [Imagawa et al., 1999]. Subsequently it was demonstrated that RGS2 may promote preadipocyte differentiation [Nishizuka et al., 2001]. RGS proteins comprise a family made up of more than 20 known members that have been implicated as negative regulators of G protein signaling [Hollinger and Hepler, 2002; Siderovski and Willard, 2005]. A number of RGS proteins have been

Grant sponsor: Taiwan National Science Council; Grant number: NSC 96-2320-B-010-025; Grant sponsor: Ministry of Education Aim for the Top University Plan; Grant number: 96A-D-D114; Grant sponsor: Department of Health of Taipei City Government; Grant number: 96002-62-087.

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Received 20 May 2008; Accepted 21 July 2008 • DOI 10.1002/jcb.21893 • 2008 Wiley-Liss, Inc.
Published online 22 August 2008 in Wiley InterScience (www.interscience.wiley.com).

purified and this has revealed that the RGS proteins act as a GTPase-activating protein (GAP) and thus inactivate the function of a specific Gα subunit [Hollinger and Hepler, 2002]. Although RGS2 is highly induced at an early stage of 3T3-L1 cell differentiation, the involved molecular regulation mechanism remains unclear.

In order to clarify the detailed regulation machinery of RGS2 expression in preadipocyte differentiation, a series of experiments were carried out to characterize the signaling pathways leading to RGS2 transcriptional activation by measuring RGS2 mRNA expression and analyzing the RGS2 promoter sequence in 3T3-L1 preadipocytes.

TABLE I. Primer Sequences Used for Semi-Quantitative RT-PCR

Gene	Sequences of forward (F) and reverse (R) primers	Size (bp)
β-actin	F: 5′-GGC ACC ACA CCT TCT ACA AT-3′ R: 5′-CGT CAT ACT CCT GCT TGC TG-3′	834
RGS2	F: 5′-GAC CCG TTT GAG CTA CTT CTT-3′ R: 5′-CCG TGG TGA TCT GTG GCT TTT TAC-3′	554
C/EBPα	F: 5′-AGG TGC TGG AGT TGA CCA GT-3′ R: 5′-CAG CCT AGA GAT CCA GCG AC-3′	238

RGS: Regulator of G protein signaling.
C/EBPα: CCAAT/enhancer binding protein α.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Western blot chemiluminescence reagent and the luciferase assay kit were purchased from PerkinElmer (Shelton, CT). Reverse transcriptase and T4 DNA ligase were obtained from Promega (Madison, WI). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). SuperFect transfection reagent and plasmid purification columns were supplied by Qiagen (Valencia, CA). The goat anti-aP2 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the mouse anti-α-tubulin monoclonal antibody was bought from Sigma Chemicals (St. Louis, MO). Anti-mouse and anti-goat IgG antibodies coupled to horseradish peroxidase were purchased from Amersham Biosciences (Piscataway, NJ). The Western blot transfer membrane immobilon-P was purchased from Millipore (Bedford, MA). Unless otherwise specified, all other chemicals and reagents used in this project were purchased from Sigma Chemicals.

CELL CULTURE

The ATCC-derived cell line NIH 3T3-L1 was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. To initiate differentiation, the medium was changed to DMEM containing 10% FBS with the inclusion of MDI, including 0.5 mM 3-isobuty-1-methylxanthine (MIX), 0.5 μM dexamethasone (DEX), and 10 μg/ml insulin (I) at 48 h post-confluence [Student et al., 1980; Smith et al., 1988].

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

After termination of differentiation treatment, the cells were washed once with 1× PBS and total RNA was prepared from the cells using Tri-Reagent (Sigma Chemicals) according to the manufacturer's instruction. The isolated RNA samples were resuspended in RNase-free diethylpyrocarbonate (DEPC)-treated water and subjected to regular semi-quantitative RT-PCR. The final cDNA yields were measured against the signal obtained from the internal standard house-keeping gene β-actin after amplification for 30 PCR cycles with appropriate setting parameters. The primer sequences are listed in Table I. The PCR products were subjected to electrophoresis on a 2% agarose gel with 1 μg/ml ethidium bromide. The DNA signal on the gel was captured and analyzed by ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA).

SUBCLONING OF THE MOUSE RGS2 5′-FLANKING SEQUENCE AND PROMOTER ACTIVITY ANALYSIS

In order to study the transcriptional regulation of the RGS2 gene during 3T3-L1 differentiation, a near 3 kb DNA fragment (−2980 to +1 of transcription start site) [Thirunavukkarasu et al., 2002] of the 5′-flanking sequence of the mouse RGS2 gene was isolated by PCR from mouse genomic DNA using specific primers (Table II) and fused with a luciferase reporter gene within a reporter vector (pGL-2B, Promega). This plasmid was then used to examine promoter activity by evaluating the luciferase activity in the cell lysates. The RGS2 promoter construct was contransfected with a control plasmid expressing β-galactosidase (driven by a CMV promoter). The detected luciferase was normalized against the β-galactosidase activity within the same sample. To identify specific signaling pathways leading to RGS2 transcriptional activation during 3T3-L1 differentiation, the full-length RGS2 promoter construct was transfected into 3T3-L1 cells. The cells were then subjected to the culture medium containing 10% FBS only or including 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (0.5 μM), insulin (10 μg/ml), or the regular MDI recipe. In addition, a variety of inhibitors of various signaling pathways were added to pinpoint the

TABLE II. Primer Sequences Used for Mouse RGS2 Promoter Cloning and Site-Directed Mutagenesis

Deletion/mutation	Primer sequences
mRGS2 promoter (−2980/+1)	F: 5′-CAG GTA CCC ACA GTT TTG GAG TTC TTT C-3′ R: 5′-TTC TCG AGA CCG CAG CTG TTT GAG GCC G-3′
Del-135 (−135/+1)	F: 5′-CAG GTA CCC TCC GCC CTC GTG CCG TCT GC-3′
Del-78 (−78/+1)	F: 5′-CAG GTA CCA CGC CTC CAG GTC CCG CCC C-3′
CCAAT mutation	WT: 5′-GTG CCG TCT GCA GCG AGC CAA TCC GCG CTC TCT TGG GGC G-3′ Mut: 5′-GTG CCG TCT GCA GCG AGC ttg gCC GCG CTC TCT TGG GGC G-3′
Sp1-A mutation	WT: 5′-GGT ACC ACG CCT CCA GGT CCC GCC CCT ATG CCG CCG CCC-3′ Mut: 5′-GGT ACC ACG CCT CCA GGT ttt GCC CCT ATG CCG CCG CCC-3′
Sp1-B mutation	WT: 5′-TCC CGC CCC TAT GCC GCC CCC CGC CTT AAA AGC CCG CGG-3′ Mut: 5′-TCC CGC CCC TAT GCC GCC ttt CGC CTT AAA AGC CCG CGG-3′

signaling mechanism(s) leading to RGS2 transcriptional induction. In order to identify the critical DNA elements mediating RGS2 transcription, the 5′-end progressive deletion mutants of the RGS2 promoter construct were obtained by restriction enzyme digestion or by PCR (primer sequences described in Table II). To further identify the critical elements within the RGS2 promoter region, site-directed mutagenesis using a QuikChange® site-directed mutagenesis kit (Stratagene, LA Jolla, CA) was adopted to generate mutation in a CCAAT element as well as in two Sp1 elements (Table II). The mutated promoter plasmid was analyzed at 12 h, 3 days and 6 days after initiating differentiation. This was aimed at helping to clarify which element(s) are required for the RGS2 transcription under acute (12 h) as well as sustained induction (day 3 and day 6). To measure luciferase activity in the cell lysates, the medium was removed and cells were washed once with 1× PBS and lysed with Glo luciferase lysis buffer (Promega). The luciferase activity in the samples was measured using a commercial luciferase assay kit and monitored by a VICTOR2 multilabel counter (PerkinElmer, Waltham, MA). Transfection efficiency was determined by measuring the β-galactosidase activity within the same sample.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts were prepared with a nuclear extraction kit (Panomics, Freemont, CA) from the treated 3T3-L1 cells and subjected to the EMSA analysis with an EMSA kit (Panomics). To clarify whether the Sp1 transcription factor was involved in binding to critical the RGS2 promoter region, Sp1 antibodies were used to clarify the interaction between the Sp1 transcription factor and the corresponding Sp1 DNA element.

STATISTICAL ANALYSIS

Experimental data were expressed as standard errors of the means (mean ± SEM) and were analyzed by one-way ANOVA, followed by the Dunett's test to compare the difference between the treatment groups and the control group. The *P* values smaller than 0.05 were considered significant.

RESULTS

DIFFERENTIATION OF 3T3-L1 CELLS AND INDUCTION OF RGS2 mRNA

C/EBPα and aP2 are two well recognized early differentiation markers during preadipocyte differentiation [Rosen and Spiegelman, 2000]; therefore to confirm that the 3T3-L1 cells underwent differentiation when induced by the regular MDI (3-isobuty-1-methylxanthine [MIX], dexamethasone [DEX], insulin [I]) differentiation recipe. Expression of aP2 protein was measured and found to be dramatically induced 2 days after the initiation of differentiation (Fig. 1A). The expression of C/EBPα mRNA during 3T3-L1 differentiation were also examined and found to be significantly induced at day 1 (Fig. 1A). Another line of evidence assuring that successful differentiation had taken place was the accumulation of fat droplets in 5-day differentiated cells, confirmed by the oil red O staining method (data not shown). Before 3T3-L1 cells were induced to undergo differentiation, a basal level of expression for RGS2 mRNA was noted (Fig. 1A,B). RGS2 mRNA

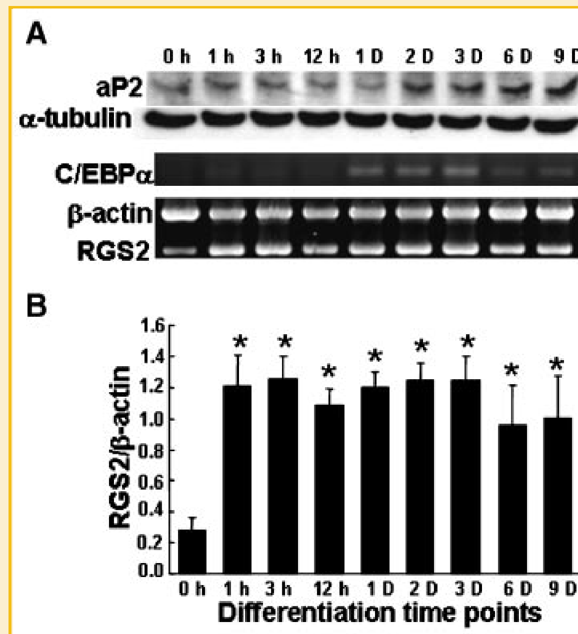

Fig. 1. Rapid induction of RGS2 mRNA during 3T3-L1 preadipocyte differentiation. After MDI treatment, cell extracts were collected at the indicated time points. A: The C/EBPα and RGS2 mRNA levels were examined by semi-quantitative RT-PCR with β-actin as an internal control. The expression of aP2 protein and an internal control α-tubulin were examined by immunoblot analysis. B: The RGS2 mRNA expression profiles were quantified and the results are expressed as mean ± SEM from three separate experiments. $^{*}P < 0.05$ compared with 0 h control.

expression was rapidly and dramatically elevated at 1 h after differentiation initiation with a fourfold increase, and this high level of RGS2 mRNA was maintained until the late stage (day 9) of differentiation (Fig. 1A,B).

TRANSCRIPTIONAL ACTIVATION OF RGS2 PROMOTER ACTIVITY DURING DIFFERENTIATION

In order to examine the transcriptional regulation of the RGS2 gene during 3T3-L1 differentiation, a 3 kb portion of the 5′-flanking sequence from the mouse RGS2 gene was isolated and analyzed for its promoter activity during 3T3-L1 differentiation. As shown in Figure 2A, luciferase activity in cells containing the RGS2 promoter plasmid was significantly induced at 3 h and the elevation remained at least until 48 h (Fig. 2A). As the RGS2 mRNA as well as the RGS2 promoter was induced by the in vitro MDI [Student et al., 1980] differentiation program (Figs. 1 and 2A), we went on to examine which of the three components of MDI was crucial to activate RGS2 promoter function. As shown in Figure 2B, the complete MDI, a combination of MIX and DEX, or of MIX and I, or MIX alone all induced RGS2 promoter function with a similar amplitude (Fig. 2B); however, a combination of DEX and I, or treatment with DEX or I alone were not able to affect RGS2 promoter activity (Fig. 2B), indicating that within the MDI treatment, MIX is the major component that stimulates RGS2 transcription. MIX is an inhibitor of cAMP phosphodiesterase (PDE), and this enzyme should produce an accumulation of intracellular cAMP [Soderling and Beavo, 2000],

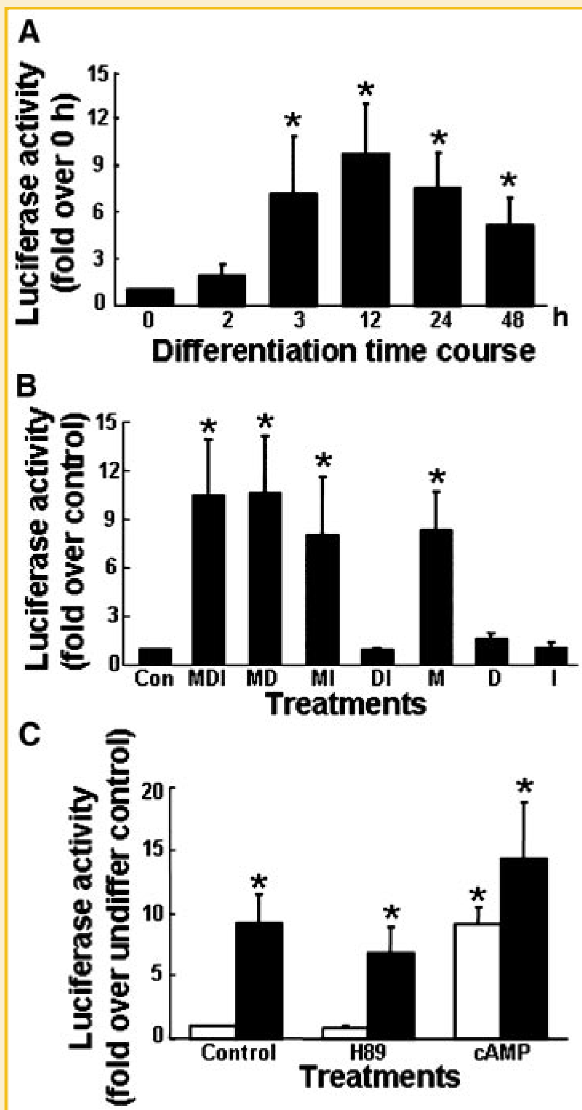

Fig. 2. Induction of RGS2 promoter activity during 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were cotransfected with mouse RGS2 promoter construct fused with the luciferase reporter gene and a reference plasmid with a CMV promoter driven β-galactosidase reporter gene. A: Confluent transfected cells were differentiated with MDI treatment and collected at the indicating time points. RGS2 promoter activity was detected by luciferase assay and normalized against the β-galactosidase activity within the same sample. The results represent the mean ± SEM of four separate experiments with triplicates in each experiment. $^{*}P < 0.05$ compared with 0 h control. B: To evaluate the contribution of MIX, DEX and insulin (I) to the induction of RGS2 promoter, confluent transfected cells were treated with various combinations of the differentiation chemicals for 12 h. The results represent the mean ± SEM of three individual experiments with triplicates in each experiment. $^{*}P < 0.05$ compared with undifferentiated control. C: To further clarify the significance of cAMP and PKA in RGS2 promoter activation, confluent transfected cells were treated or not treated with MDI with the inclusion of H89 (5 μM) or 8-bromo-cAMP (1 mM) for 12 h. The white columns represent the groups with no differentiation and the black columns represent the groups with differentiation. The results represent the mean ± SEM of four separate experiments with triplicates in each experiment. $^{*}P < 0.05$ compared with the undifferentiated control group.

and subsequently often transmits signals by activating protein kinase A (PKA) and its downstream signal transduction [Soderling and Beavo, 2000]. In order to further clarify whether PKA is acting downstream of cAMP to mediate RGS2 transcriptional activation, a PKA inhibitor H89 and a synthetic form of cAMP, 8-bromo-cAMP were used to treat the 3T3-L1 cells with or without the inclusion of MDI. In the absence of MDI, H89 had no effect on the RGS2 promoter activity (Fig. 2C), and 8-bromo-cAMP treatment generated a similar induction as the MDI treatment (Fig. 2C). Under treatment with MDI, 8-bromo-cAMP did not cause a further increase in the RGS2 promoter activity and the addition of H89 did not affect the MDI-induced RGS2 promoter activation (Fig. 2C). In addition, we also examined whether any other protein kinase might potentially be involved in the MDI-mediated RGS2 transcriptional activation during 3T3-L1 differentiation. A panel of protein kinase inhibitors, including p38 MAPK inhibitor (SB203580), ERK inhibitor (PD98059), JNK inhibitor (SP600125), JAK inhibitor, PKA inhibitor (H89), PKC inhibitor (Calphostin C), PI 3-kinase inhibitor (LY294002, Wortmannin), Src kinase inhibitor (SU6656), and AMPK inhibitor (Compound C) were used to evaluate RGS2 promoter activity under MDI treatment. Promoter activity when the cells were treated with PD98059, LY294002, or Wortmannin and MDI tended to be higher than with MDI alone; however, none of the tested inhibitors appeared to significantly affect MDI-induced RGS2 promoter activation (Fig. 3).

IDENTIFICATION OF THE CRITICAL RESPONSIVE ELEMENT WITHIN THE RGS2 PROMOTER REGION

In order to identify the critical DNA element(s) mediating RGS2 transcription during differentiation, the RGS2 promoter sequence was progressively deleted from the 5′-end by restriction enzyme digestion as well as by PCR and then analyzed in MDI-treated cells. As shown in Figure 4A, when the promoter sequence was truncated down to as short as 135 bp, it retained full-inducibility by MDI (Fig. 4A), suggesting that the critical DNA responsive element is localized within this 135 bp region. Within this 135 bp region, a CCAAT element resides between −115 and −95. As the C/EBPα, C/EBPβ, and C/EBPδ have been well recognized as important transcription factors in preadipocyte differentiation [Rosen and Spiegelman, 2000; Rosen, 2005], the CCAAT element was mutated and evaluated for the promoter activity. Surprisingly, the CCAAT mutant exhibited a similar promoter activity to that of the wild-type 135 bp promoter sequence (Fig. 4B). Therefore the 135 bp sequence was further truncated down to a 78 bp by PCR and analyzed for the promoter activity. The 78 bp region retained full-inducibility by MDI (Fig. 5A) as compared with the full-length 3 kb sequence and with the 135 bp sequence (Fig. 5A). These results also confirmed the finding in Figure 4 that the CCAAT element is not an essential element for control of RGS2 transcription. Within the 78 bp region, there are two separate consensus Sp1 DNA elements [Lania et al., 1997]. These two potential Sp1 sites were mutated alone or in combination and evaluated for RGS2 promoter activity. When the first Sp1 site (Sp1-A) was mutated, inducibility by MDI treatment was completely blocked (Fig. 5B) and at the same time, the basal RGS2 promoter activity was also significantly reduced (Fig. 5B). Furthermore, mutation within the second Sp1 (Sp1-B) site did not

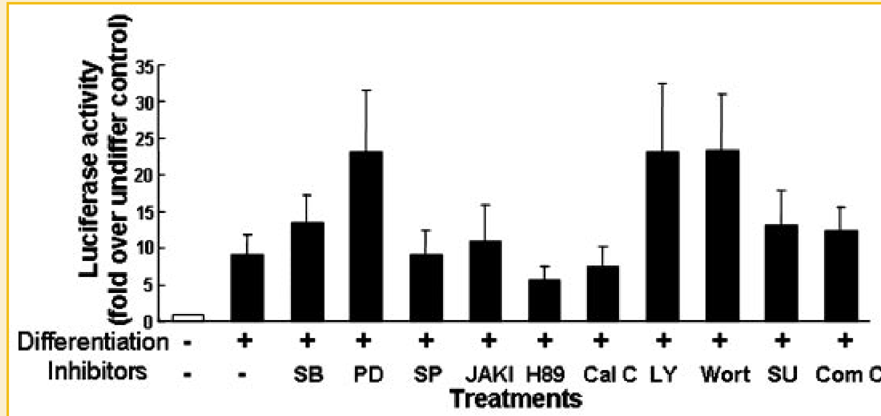

Fig. 3. Effect of different protein kinase inhibitors on RGS2 promoter activation. 3T3-L1 preadipocytes were cotransfected with the full-length mouse RGS2 promoter construct fused with the luciferase reporter gene and the reference plasmid CMV-β-galactosidase. Confluent transfected cells were differentiated with MDI treatment and cotreated with various protein kinase inhibitors, namely SB (SB203580, 5 μM); PD (PD98059, 10 μM); SP (SP600125, 10 μM); JAK I (JAK inhibitor, 10 μM); H89 (5 μM); Cal C (Calphostin C, 10 μM); LY (LY294002, 10 μM); Wort (Wortmannin, 10 μM); SU (SU6656, 10 μM) and Com C (Compound C, 5 μM), for 12 h. RGS2 promoter activity was detected by luciferase assay and normalized against β-galactosidase activity. The results represent the mean ± SEM of three separate experiments with triplicates in each experiment.

change basal or MDI-induced promoter activity (Fig. 5B). Mutation in both Sp1 sites resulted in a similar effect to that which occurred with the single mutation in the Sp1-A site (Fig. 5B). These results suggested that the Sp1-A site rather the Sp1-B site is a critical element mediating RGS2 transcription during 3T3-L1 differentiation. As we observed in Figure 1, RGS2 mRNA remained high until the late stage (day 9) of differentiation, we therefore also looked into the regulation of RGS2 promoter during these later stage of 3T3-L1 differentiation, specifically on days 3 and 6. The full-length, wild-type 135 bp, 135 bp with CCAAT mutation, wild-type 78 bp, 78 bp with the Sp1-A mutation, 78 bp with the Sp1-B mutation, and 78 bp with the Sp1-A and Sp1-B double mutation were examined at day 3

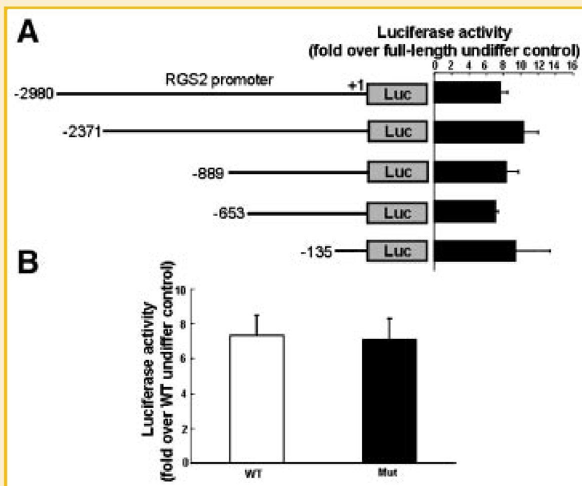

Fig. 4. Nonessential role of the CCAAT box in RGS2 promoter activation during 3T3-L1 preadipocyte differentiation. A: Sequential deletion constructs of the RGS2 promoter were obtained by enzyme digestion or PCR, and fused with luciferase reporter gene. Confluent transfected 3T3-L1 cells were differentiated with MDI treatment and collected 12 h after differentiation. Four separate experiments were performed. B: The 135 bp RGS2 promoter sequence with the CCAAT box was mutated by site-directed mutagenesis using the primers described in Table II. Confluent 3T3-L1 cells were cotransfected with the plasmid bearing wild-type (white column) or the mutant CCAAT element (black column) and the β-galactosidase reference plasmid. The results represent the mean ± SEM of three separate experiments with triplicates in each experiment.

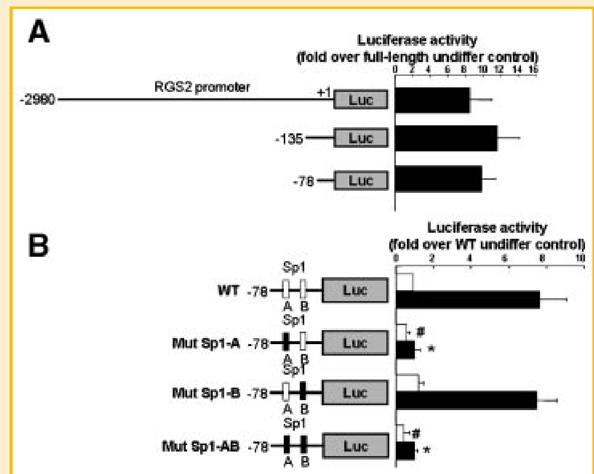

Fig. 5. Critical role of the Sp1 site in the RGS2 promoter activation during 3T3-L1 differentiation. A: The full-length, the 135 bp and the further deleted 78 bp RGS2 promoter sequence were analyzed as described. Three independent experiments were performed. B: The two Sp1 sites, the Sp1-A site and the Sp1-B site, within the 78 bp RGS2 promoter sequence, were mutated by site-directed mutagenesis either alone or in combination. The promoter constructs were transfected into 3T3-L1 cells and induced to differentiate for 12 h. The white columns represent the groups with no differentiation and the black columns represent the groups with differentiation. The results represent the mean ± SEM of three separate experiments with triplicates in each experiment. $^{*}P < 0.05$ compared with the WT differentiated group; $^{\#}P < 0.05$ compared with the WT no differentiation group.

and at day 6 after MDI treatment. At both day 3 and day 6, the shortest 78 bp sequence retained comparable promoter activity with the full-length promoter (Fig. 6A,B) and only the Sp1-A site mutation significantly reduced both basal and MDI-induced RGS2 promoter activity (Fig. 6A,B).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) OF THE Sp1 RESPONSIVE ELEMENT

In order to delineate the interaction of Sp1 factors with the Sp1 responsive element, nuclear proteins were isolated at different time intervals from 3T3-L1 cells that were untreated or treated with MDI. Incubation of these nuclear proteins with labeled oligonucleotide corresponding to the Sp1 element gave a distinct band at the 0, 1, 3, 6, and 12 h time points (Fig. 7A). At all time points, the band density was only marginally decreased by the preincubation of nuclear extracts with antibodies against Sp1 (Fig. 7A). This indicated that the Sp1 protein were continuously present in 3T3-L1 cells before and after differentiation initiation. However, when excess wild-type or mutant Sp1-A oligonucleotides was included during complex formation, the distinct band was blocked completely (Fig. 7B). With the inclusion of Sp1 antibodies, the density of the distinct band was not substantially affected (Fig. 7B). To clarify whether the Sp1 expression corresponds with the induction of RGS2 promoter activation profile, expression of Sp1 protein was analyzed in the total lysates from the same time points as in the Figure 1. Expression of Sp1 seemed to be consistent before and after 3T3-L1 differentiation (Fig. 7C). Although not shown, similar results were noted by analyzing the nuclear proteins from 3T3-L1 cells that had been induced to differentiate for the same time points (data not shown).

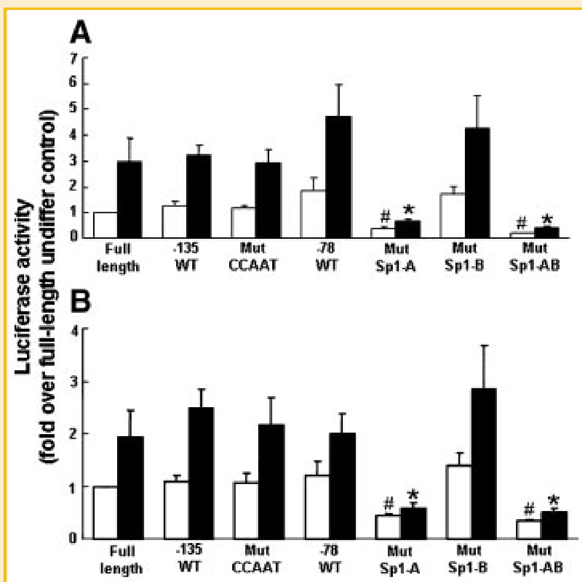

Fig. 6. Essential role of the Sp1 site in the RGS2 promoter activity at 3 days or 6 days after 3T3-L1 differentiation. The RGS2 promoter sequence, with the full-length promoter, the wild-type 135 bp promoter, the 135 bp promoter with the CCAAT mutation, the wild-type 78 bp promoter, the 78 bp promoter with a mutation in the Sp1-A site, the 78 bp promoter with a mutation in the Sp1-B site or the 78 bp promoter with mutation in both the Sp1-A and Sp1-B site in combination, were transfected into 3T3-L1 cells and cells were induced to differentiate for 3 days (A) or 6 days (B). The white columns represent the undifferentiation groups and the black columns represent the differentiation groups. The results represent the mean ± SEM SEM of four separate experiments with triplicates in each experiment. $^{*}P < 0.05$ compared with the full-length undergoing differentiation; $^{\#}P < 0.05$ compared with the full-length not undergoing differentiation.

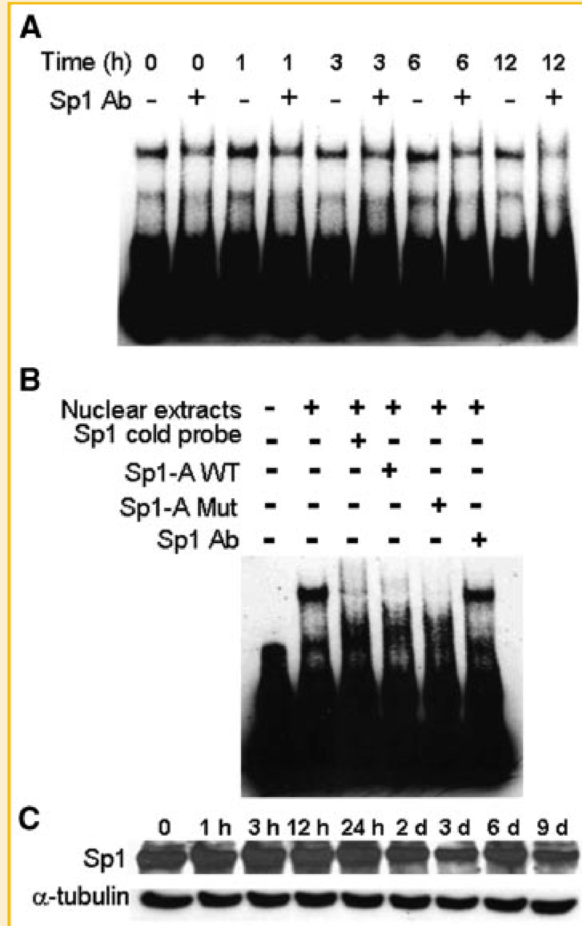

Fig. 7. Electrophoretic mobility shift assay (EMSA) of the Sp1 site with 3T3-L1 nuclear extracts. The confluent 3T3-L1 cells were differentiated with MDI treatment and collected at the indicated time points. Nuclear extracts were obtained by nuclear extraction kit and analyzed by EMSA assay. A: Specific Sp1 antibodies were included in the preincubation before the addition of the Sp1 probe. B: The analysis of the nuclear extracts from 12 h differentiated 3T3-L1 cells was carried out with 50-fold excesses of the control Sp1 cold probe, wild-type Sp1 oligonucleotide or mutant Sp1-A oligonucleotides as controls to examine the interaction between Sp1 site and transcription factors in the samples. C: To examine the expression levels of Sp1 protein in 3T3-L1 cells during the differentiation process, the confluent cells were differentiated with MDI treatment and collected at the indicated time points. The total protein extracts were obtained and analyzed by immunoblot assay. Results shown represent three independent experiments.

DISCUSSION

The results of the present study demonstrate that RGS2 mRNA expression is immediately induced in 3T3-L1 after initiating

differentiation and its expression remained consistently high until the late stage of differentiation. Transcriptional induction of RGS2 gene is mediated by a cAMP-dependent, but not PKA-dependent pathway. Additionally, a Sp-1 response element within the RGS2 promoter sequence is responsible for RGS2 transcriptional activation, from immediate early stage to late stage in differentiating 3T3-L1 cells.

In order to understand adipogenesis, in vitro culture system is the most used experimental model [Taylor and Jones, 1979; Ntambi and Young-Cheul, 2000], and preadipocyte 3T3-L1 cells that have finished the commitment step to adipocyte lineage and will mature into adipocytes with appropriate induction [Ntambi and Young-Cheul, 2000; Otto and Lane, 2005] are perhaps the best studied model. By using the 3T3-L1 differentiation system we found that there was a basal expression RGS2 mRNA in 3T3-L1 cells before differentiation initiation (Fig. 1), and an immediate dramatic elevation at 1 h after differentiation was noted. Such rapid induction of RGS2 mRNA expression is consistent with several other studies in a variety of cell types [Ko et al., 2001; Thirunavukkarasu et al., 2002; Gold et al., 2003; Kim et al., 2006; Wu et al., 2008]. There appears to be a basal expression of RGS2 mRNA before differentiation induction; however RGS2 expression increased promptly and dramatically at the onset of differentiation and the level of expression remained consistently high until the late stage of differentiation. However, induction of RGS2 expression in several other cell types seems to be rapid and transient [Ko et al., 2001; Thirunavukkarasu et al., 2002; Gold et al., 2003; Kim et al., 2006; Wu et al., 2008]. Such a persistent expression profile of RGS2 may imply some function significance of RGS2 along the adipocyte differentiation and this should deserve further investigation.

It has been previously shown that treatment with cAMP is sufficient to induce adipocyte differentiation in vitro [Reusch et al., 2000], suggesting that a cAMP-mediated signaling pathway plays a critical role in adipocyte maturation and adipose functions [Reusch et al., 2000]. Apart from this, our study revealed that cAMP alone was sufficient to induce RGS2 expression during preadipocyte differentiation (Fig. 2), suggesting that cAMP-induced adipocyte differentiation [Reusch et al., 2000] is likely to at least partly regulate through RGS2. Similarly, it has been previously shown in murine osteoblasts that cAMP is responsible for the induction of RGS2 expression by parathyroid hormone [Tsingotjidou et al., 2002]. In our study, DEX treatment alone did not activate RGS2 promoter; however DEX treatment has been shown in a previous study to induce RGS2 mRNA expression [Imagawa et al., 1999]. It is possible that the DEX acted at the post-transcription, but not at the transcription stage of RGS2 expression. The mitogen activated protein kinases (MAPKs) ERK, p38 and JNK are also intracellular signaling pathways that play a pivotal role in many cellular events, such as proliferation and differentiation and their well-tuned regulation and significance in adipocyte differentiation has also been demonstrated [Bost et al., 2005]. In addition, the importance of PI 3-kinase in adipocyte differentiation has also been reported using PI 3-kinase inhibitors to block the differentiation of adipogenic cell lines [Xia and Serrero, 1999]. However, when several kinase inhibitors affecting the above signaling pathways were tested in this study, none of them appeared to significantly affect RGS2 transcription. This suggests that RGS2 transcriptional activation in differentiating 3T3-L1 cells was likely to be under a unique type of regulation by cAMP.

By dissecting the mouse RGS2 promoter sequence, we primarily identified an essential Sp1 element for RGS2 transcription. Classically, the Sp1 site is bound by members of the transcription factor Sp family, which is known to contains at least eight members, namely Sp1 to Sp8 [Bouwman and Philipsen, 2002]. In addition, the Sp1 element has been demonstrated to be involved in the regulation of several adipocyte differentiation-related genes [Tang et al., 1999; Jiang and Lane, 2000; Zhu and Liao, 2000; Barth et al., 2002; Schaffler et al., 2004; Shimba et al., 2004; Wada, 2007]. Among these genes, the Sp1 transcription factor may acts as either a positive or a negative regulator. In our study, when the first, but not the second Sp1 site within the RGS2 promoter sequence was mutated, RGS2 promoter activity was completely abolished, indicating that the first Sp1 site is the essential element mediating RGS2 gene transcription. The most common Sp1 consensus element is 5′-CCCCGCCCCG-3′ [Letovsky and Dynan, 1989]. Within the shortest 78 bp mouse RGS2 promoter sequence retaining full-inducibility by MDI, the Sp1-A site (5′-GTCCCGCCCC-3′) seems to be less similar to the consensus sequence than the Sp1-B site (5′-CCGCCCGCC-3′). Therefore, it is intriguing that Sp1-A rather than Sp1-B is essential for mediating MDI-induced RGS2 transcription. Recently it was reported that when the −391 site of a human RGS2 promoter region was changed from a C nucleotide to a G nucleotide, RGS2 expression was changed [Freson et al., 2007]. Indeed, the Sp1 site covers the −391 site, suggesting the importance of the Sp1 site in the regulation of human RGS2 gene [Freson et al., 2007]. In addition, by EMSA assay the distinct band formed with the Sp1 element did not appear to be disrupted by the inclusion of specific Sp1 antibodies (Fig. 7), and the expression of Sp1 did not seem to be different before and through the 3T3-L1 differentiation (Fig. 7), suggesting that during the regulation of RGS2 transcription, while preadipocyte differentiation is taking place, there might be the involvement of other transcription factors or other novel regulation mechanisms via the Sp1 element. For example, MDM2, an inhibitor of p53 transcription factor, has been shown to induce NFκB p65 promoter activation by direct binding to the consensus Sp1 sites [Gu et al., 2002].

The C/EBP proteins have been well recognized to play important roles in preadipocyte differentiation by acting as early regulators, which induce downstream players that are involved in adipogenesis [Rosen, 2005]. In our study, RGS2 expression was induced at an even earlier stage of differentiation, namely 1 h, and indeed the CCAAT element within the RGS2 promoter was not important mediating RGS2 transcription (Figs. 4–6). This supports the idea that the involvement of RGS2 in 3T3-L1 differentiation is probably required at an earlier stage than even the C/EBP proteins. According to several studies, adipocyte differentiation and maturation are strongly influenced by the action of numerous hormones and extracellular factors, some of which may act together with G proteins [MacDougald and Mandrup, 2002], and in fact several GPCRs have also been detected in preadipocytes, adipocytes, and adipose tissues [Guest et al., 1990; Betuing et al., 1997; Collins and Surwit, 2001; Monjo et al., 2005; Gotoh et al., 2007]. Differential expression of various G protein Gα subtypes in preadipocytes and

matured adipocytes have been reported [Denis-Henriot et al., 1996a,b] and Gα12 and Gαi2 have been shown to be involved in preadipocyte differentiation [Gordeladze et al., 1997; Denis-Henriot et al., 1998]. All of these studies suggest that G protein signaling is an important part in adipocyte differentiation. RGS2 has been demonstrated to be a negative regulator of G protein signaling [Willars, 2006], including the Gαq-mediated signaling pathway [Anger et al., 2004]. Meanwhile, two specific GPCR agonists lysophosphatidic acid (LPA) and PGF2α have been shown to inhibit preadipocyte differentiation [Simon et al., 2005; Liu and Clipstone, 2007]. Therefore, induction of RGS2 expression in the immediate early stage of preadipocyte differentiation is likely to halt the signaling of Gαq, which can be mediated by various GPCR activations, for example the PGF2α receptor [Watanabe et al., 1995; Wu et al., 2008], which often activates the Gαq, resulting in preadipocyte differentiation [Quarles et al., 1993].

In studies of preadipocyte differentiation, RGS2 gene has been demonstrated to be immediately induced in differentiating 3T3-L1 cells [Imagawa et al., 1999] and has also been shown to promote preadipocyte differentiation in the presence of PPARγ [Nishizuka et al., 2001]. As the high and immediate induction of RGS2 expression appears in the earlier stage [Imagawa et al., 1999] than the induction of C/EBP and PPAR families, suggesting that RGS2 may play a crucial role in the very early stage of differentiation. Thus, to fill the gap of our knowledge of the very early stage of preadipocyte differentiation and its regulation, RGS2 appears to be a good candidate to carry on further investigation. In this study, we adopted an in vitro differentiation model in 3T3-L1 culture system to clarify the precise induction profile of RGS2 expression, the transcriptional regulation of RGS2 with the involvement of an essential Sp1 element within the RGS2 promoter and the involving cAMP-dependent pathway. The present study has provided a starting point to expand our knowledge of the regulation of RGS2 expression toward its functions in adipocyte differentiation and maturation and this should potentially provide valuable information to improve human health as well as illuminate the development of treatments for various obesity related diseases.

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