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Integrator complex plays an essential role in adipose differentiation

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ABSTRACT

The dynamic process of adipose differentiation involves stepwise expressions of transcription factors and proteins specific to the mature fat cell phenotype. In this study, it was revealed that expression levels of IntS6 and IntS11, subunits of the Integrator complex, were increased in 3T3-L1 cells in the period when the cells reached confluence and differentiated into adipocytes, while being reduced to basal levels after the completion of differentiation. Suppression of IntS6 or IntS11 expression using siRNAs in 3T3-L1 preadipocytes markedly inhibited differentiation into mature adipocytes, based on morphological findings as well as mRNA analysis of adipocyte-specific genes such as Glut4, perilipin and Fabp4. Although Pparγ2 protein expression was suppressed in IntS6 or IntS11-siRNA treated cells, adenoviral forced expression of Pparγ2 failed to restore the capacity for differentiation into mature adipocytes. Taken together, these findings demonstrate that increased expression of Integrator complex subunits is an indispensable event in adipose differentiation. Although further study is necessary to elucidate the underlying mechanism, the processing of U1, U2 small nuclear RNAs may be involved in cell differentiation steps.

1. Introduction

White adipose tissue (WAT) is a major energy reserve in higher eukaryotes, and storing triacylglycerol in periods of energy excess and its mobilization during energy deprivation are its primary roles. Understanding the mechanisms of adipogenesis is of major relevance to human disease, as adipocyte dysfunction is a major contributor to metabolic disease in obesity [1,2]. For the past two decades, many intensive studies have revealed the process of stepwise expressions of transcription factors and proteins which take place during the transition from preadipocytes into mature adipocytes. Briefly, growth arrested 3T3-L1 preadipocytes treated with differentiation inducers immediately express C/ebpβ, and C/ebpβ triggers transcription of Pparγ and C/ebpα, which in turn induce adipocyte-specific genes such as Glut4, perilipin and Fabp4 in a coordinate fashion [3–5].

While the aforementioned transcriptional cascade itself has been largely clarified, much attention is now being given to newly identifying the proteins which regulate and/or initiate these processes. For example, upregulation of Pin1 is essential for the differentiation of preadipocytes into adipocytes, although it is still unclear how Pin1 affects the upregulation of adipocyte-specific gene expressions [6,7]. In this study, we attempted to screen the proteins associated with AS160 reportedly playing a role in the process from Akt activation to the translocation of GLUT4 to the cell surface [8] and IntS6 was identified as one of these proteins. IntS6 is one of the components constituting the Integrator complex, which plays critical roles in the 3′ end processing of U1 and U2 small nuclear (sn) RNAs. Subsequently, we planned to perform experiments examining whether or not this association is critical for GLUT4 translocation. However, unexpectedly, it was revealed that gene silencing of IntS6 using small interfering RNAs (siRNA) suppresses the adipose differentiation of 3T3-L1 preadipocytes. Therefore, we instead focused on this effect, and herein show the critical role of the Integrator complex in adipose differentiation.

2. Materials and methods

2.1. Antibodies and adenoviruses

Anti-IntS6 antibody was generated by immunization of rabbits with the glutathione S-transferase-fused COOH-terminal 98 amino acids of IntS6. Anti-actin antibody was purchased from Fabgennix

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(Mississauga, ON, Canada). anti-IntS11 antibodies were from Abcam. Anti-GLUT4, anti-C/EBPα, anti PPAR-γ, anti-Fabp4, anti C/EBPδ and anti-perilipin were from Cell Signaling Technology. Anti-C/EBPβ was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies were purchased from GE Healthcare (Buckinghamshire, UK). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Recombinant adenoviruses encoding mouse PPARγ2 and green fluorescent protein (GFP) were prepared according to the instruction manual of the Adenovirus Dual Expression Kit (TaKaRa Bio). Adenovirus encoding GFP alone served as a control.

2.2. Cell culture and induction of differentiation

3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (vol/vol) calf serum. After the cells reached 100% confluence, the culture medium was changed to DMEM containing 10% FBS and 4500 mg/L of D-glucose. After two days (designated day 0), the cells were induced to differentiate with DMEM containing 10% FBS, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methyl-xanthine, and 1 μg/ml insulin. After two days, the cells were maintained in DMEM supplemented with 10% FBS and 1 μg/ml insulin until day 4, after which DMEM containing 10% FBS and 4500 mg/L of D-glucose was added daily.

2.3. Oil red O staining

3T3-L1 preadipocytes which had differentiated for 6 days were subjected to visualization of intracellular lipid droplets by oil red O staining. Cells were washed three times with phosphate-buffered saline (PBS) and then fixed for 2 min with 3.7% formaldehyde. Oil red O (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 1 h at room temperature. Cells were washed with water, and the stained fat droplets in the cells were then visualized by light microscopy.

2.4. Western blot analysis

The 3T3-L1 cells at different time-points after induction were rinsed twice with PBS, and then lysed in Laemmli buffer (containing 4% SDS 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris–HCl). Equal amounts of protein lysates were separated by SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membranes in a transfer buffer consisting of 20 mM Tris–HCl, 150 mM glycine and 20% methanol.

On the other hand, to detect the large Integrator complex consisting of many subunits, the blue native-polyacrylamide gel electrophoresis (BN-PAGE) method was adopted. In order to prepare samples for BN-PAGE analysis, cells were washed with ice-cold PBS and lysed on ice with 1% digitonin-buffer (50 mM Bis–Tris–HCl pH 7.2, 1% digitonin, 50 mM NaCl, 10% glycerol, 0.001% Ponceau S) or 1% Triton ×-100-buffer (50 mM Tris–HCl pH 7.5, 1% Triton ×-100, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA). Before use, each buffer was mixed with 500 μM Na_3VO_4, 100 kallikrein-inactivating units (KIU)/ml aprotinin, 20 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 5 μg/ml pepstatin. The lysates were centrifuged at 15,000*g* for 10 min at 4 °C, and 10 μg of the protein of each supernatant were mixed with BN-PAGE sample buffer (Invitrogen) and Coomassie brilliant blue G-250, according to the manufacturer's recommended protocols.

The membranes were blocked with 3% nonfat dry milk or 5% BSA in Tris-buffered saline with 0.1% Tween 20 and incubated with specific antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen–antibody interactions were visualized by incubation with ECL chemiluminescence reagent (GE Healthcare).

2.5. Small interfering RNA transfection

The siRNAs against IntS6 and IntS11 were purchased from Invitrogen (Stealth/siRNA duplex oligoribonucleotides), and the transfection of these siRNAs was performed using Lipofectamine RNAi Max (Invitrogen) according to the manufacturer's instructions. After 3T3-L1 preadipocytes had been plated on collagen-I coated dishes, cells were cultured to 50–70% confluence in DMEM containing 10% FBS. Then, the transfection reagents were added to the dishes to give a final concentration of 50 nM of siRNA. Transfection was consistently performed 4 days prior to the induction of adipocyte differentiation.

2.6. RT-PCR and real-time quantitative PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using a verso cDNA synthesis kit (Thermo Scientific). Quantitative PCR was performed using the Opticon Monitor (version 3; Bio-Rad). Cycling conditions comprised a 3-min denaturation step at 95 °C, followed by 40 cycles of denaturation (95 °C for 15 s), annealing (60 °C for 30 s), and extension (72 °C for 30 s). After amplification, melting curve analysis was performed. Each sample was amplified in triplicate. The primer sets for mouse genes were as follows.IntS6

For 5′: GCTGGATGGAAAGAAAACCA3′
Rev 5′: TTCTTCCCTGCCCATAGTTG3′

C/ebpβ

For 5′: CAAGCTGAGCGACGAGTACA3′
Rev 5′: AGCTGCTCCACCTTCTTCTG3′

Pparγ2

For 5′: TGGGTGAAACTCTGGGAGATTC3′
Rev 5′: GAGAGGTCCACAGAGCTGATTCC3′

C/ebpα

For 5′: TGGACAAGAACAGCAACGAG3′
Rev 5′: CCTTGACCAAGGAGCTCTCA3′

Glut4

For 5′: CAGATCGGCTCTGACGATG3′
Rev 5′: GGCATTGATAACCCCAATGT3′

Fabp4

For 5′: CATCAGCGTAAATGGGGATT3′
Rev 5′: TCGACTTTCCATCCCACTTC3′

Perilipin

For5′: GATCGCCTCTGAACTGAAGG3′
Rev5′: CTTCTCGATGCTTCCCAGAG3′

3. Results

3.1. Elevated expressions of IntS6 and IntS11 during the early phase of adipose differentiation in 3T3-L1 cells

IntS6 was identified as one of the candidates for binding to AS160 [8], by yeast two hybrid screening. Unfortunately, no endogenous associations between IntS6 and AS160 were observed, though we examined the expression of IntS6 during adipose differentiation of 3T3-L1 cells. The time-dependent expression patterns