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Differential Expression of the Adipocyte Amino Acid Transporter Is Transactivated by SP1 and SP3 during the 3T3-L1 Preadipocyte Differentiation Process

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It was identified that a Sp1-like *cis*-element in the adipocyte amino acid transporter gene (AAAT) promoter is the major *cis*-motif for its induced expression during the 3T3-L1 preadipocyte differentiation process. Electrophoretic mobility shift analysis of this *cis* DNA element showed that the transcription factors binding to this sequence were Sp1 and Sp3. Protein analysis of Sp1 and Sp3 in nuclear extracts from 3T3-L1 cells at various differentiation stages indicated that these two transcription factors existed in noninduced 3T3-L1 preadipocytes, but they did not bind to the AAAT promoter element with high affinity. They were activated after differentiation induction. It was further demonstrated that dephosphorylation of Sp1 increased its binding affinity to this inducible AAAT promoter element.

Key Words: **adipocyte differentiation; Sp1; Sp3; amino acid transporter; 3T3-L1 adipocyte; Sp1 dephosphorylation.**

Sp1 is a zinc finger type of transcription factor, which binds to GC box in gene promoter (1). Sp3, often binding to the Sp1 site, is a member of Sp transcription factor family. Sp1 has traditionally been thought to be important in activating constitutively expressed genes. Many recent findings have indicated that Sp1 also participates in regulation of many tissue-specific or inducible genes expression (2–9). Compared to Sp1, the function of Sp3 is less clear. It could be either activator or a repressor depending on gene promoters (10–12). There is also report that Sp3 plays a repressive role by competing with Sp1 for the binding site (13). The role that Sp1 and Sp3 play during adipocyte differentiation induction is not clear. It has been reported that Sp1 and a Sp1-like GC rich motif suppresses fatty acid synthase gene expression in adipocytes of lean rat but not obese rat (3). Another study has indicated that Sp1 may be negatively involved in the C/EBPα gene expression in 3T3-L1 preadipocyte (14). Beside these reports, Sp1 is also indicated to activate acetyl-CoA carboxylase gene in 30A5 preadipocyte (2, 15). Thus, Sp1 functions as either a repressor or an activator in expression regulation of these adipogenic genes.

Our previous study indicated that the major *cis* promoter element of AAAT gene, an adipocyte amino acid transporter preferentially expressed in 3T3-L1 adipocyte and in mouse adipose tissue (16), is a GC-rich Sp1-like motif. Mutation or deletion of this *cis*-element lead to completely inactivation of the differentially inducible promoter function. To understand the *trans*-activator for AAAT promoter, we analyzed the proteins bound to this *cis* promoter element. In the present paper, we report that transcription factors Sp1 and Sp3 are the major *trans*-activators for AAAT promoter, and their binding to this Sp1-like *cis*-motif is differentially induced. In addition, dephosphorylation of Sp1 increases the binding of Sp1 to this DNA motif in AAAT gene promoter.

MATERIALS AND METHODS

Materials. Poly(dI–dC) (dI–dC) was from Amersham–Pharmacia Biotech. Anti-Sp1 and Sp3 antibodies were from Santa Cruz Biotechnology.

Cell culture. The 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and allowed to reach confluence. Differentiation of two-day post-confluent preadipocytes (designated as day 0) was initiated with 1 μg/ml insulin, 1 μM dexamethasone (DEX) and 0.5 mM 3-iso-butyl-1-methylxanthine (MIX) in DMEM supplemented with 10% fetal bovine serum (17, 18). After 48 h (day 2), the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 μg/ml insulin for additional 48 h, and the cells were then fed every other day with DMEM containing 10% fetal bovine serum. The cytoplasmic triglyceride droplets are visible by day 4 and the cells are fully differentiated by day 6.

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Nuclear extracts and electrophoretic mobility shift and supershift analysis. Nuclear extracts from 3T3-L1 cells were prepared using urea extraction method. 3T3-L1 cell monolayers were washed twice with cold PBS and once with hypotonic lysis buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate, 30 mM β-glycerol phosphate and 2 μl/ml protease inhibitors cocktail 1 and 2 (PIC 1 and 2) (19). Cells were then scraped from plate into the same hypotonic buffer and incubated on ice for 5 min. NP-40 was added to the cell suspension to a final concentration of 0.1% for preadipocytes and 0.15% for adipocytes. After homogenizing the cells, nuclei were pelleted by centrifuging at 500*g* for 5 min. The nuclear pellet was then washed once with hypotonic buffer and once with nuclear storage buffer containing 40% glycerol, 50 mM Tris–HCl, pH 8.0, 3 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate, 30 mM β-glycerol phosphate and 2 μl/ml PIC 1 and 2. The washed nuclei were pelleted by centrifugation at 6000*g* for 20 s and resuspended in 10 pellet volumes of extraction buffer containing 1.1 M urea, 0.33 M NaCl, 1.1% NP-40, 17.5 mM Hepes, pH 7.6, 1.1 mM DTT, 1 mM sodium vanadate, 30 mM β-glycerol phosphate and 2 μl/ml PIC 1 and 2. After incubated on ice for 30 min, the mixture was centrifuged at 12,000*g* for 10 min. The supernatant was collected and glycerol was added to final concentration of 10%. The nuclear extract was aliquoted, quick-frozen and stored at −80°C. The protein concentration of the nuclear extract was determined by Lowry method.

Nuclear extract (2 μg) was incubated with 5 μg Poly(dI–dC) (dI–dC) in 20 μl binding buffer containing 10 mM Tris–HCl, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol and 5 mM $MgCl_2$ on ice for 10 min. Approximately 1–2 $\times$ 10^4 cpm (0.5–1 ng) of ^{32}P end-labeled double-strand oligonucleotide or DNA fragment was added to the preincubated nuclear extract mixture and then incubated at room temperature for 20 min. DNA–protein complexes were resolved on 4% polyacrylamide gels in 1$\times$ gel buffer (25 mM Tris–HCl, pH 8.3, 190 mM glycine, and 1 mM EDTA). The gel was dried and autoradiographed. For competition experiments, usually 50- to 100-fold excess unlabeled competitor DNA was added to the reaction mixture at the same time that the labeled DNA fragment was added. All the other steps were the same. For supershift experiments, 0.1 μg Sp1 or Sp3 antibody was added to the reaction mixture and incubated on ice for 10 min before the addition of labeled probe.

Sequences of oligonucleotide probes for EMSA are P1, 5′-TTCAAGTCCCGCCCTCCGCTTT-3′ corresponding to the sequence from −65 to −44 of AAAT promoter; Mp1, 5′-TTCAAGTCaataCCTCCGCTTT-3′; Mp2, 5′-TTCAAGTC--CCTCCGCTTT-3′; Mp3, 5′-TTCccagCCCGCCCTCCGCTTT-3′; and Sp1, 5′-ATTCGATCGGGGCGGGGCGAG-3′.

Western immunoblots. Equal amount nuclear extracts (20 μg protein) prepared from 3T3-L1 cells at various differentiation stages (days 0, 2, 4, and 6) were subjected to 8% SDS–PAGE and then transferred to Immobilon-P membrane. The membrane was then immunoblotted with antibody against Sp1 or Sp3 and visualized by ECL.

Dephosphorylation of nuclear extract. Nuclear extract from noninduced day 0 3T3-L1 preadipocytes were prepared as described above except that vanadate and β-glycerol phosphate was removed from the buffer. Dephosphorylation reaction was carried out at 1 or 2 units calf intestinal alkaline phosphatase with 10 μg nuclear extract in EMSA reaction buffer at 30°C for 30 min and then 10 min on ice. The EMSA reaction was as described above.

RESULTS

Analysis of proteins bound to AAAT promoter. From the AAAT promoter region (GenBank No. AF080267), a GC-rich Sp1-like DNA motif at position of −65 to −46, 5′-TTCAAGTCCCGCCCTCCGCTTT-3′, was identified as the major *cis*-element responsible for activating AAAT gene during 3T3-L1 differentiation induction. After locating the essential *cis*-element for AAAT promoter, attempts were made to identify the cognate *trans*-protein factors. To verify the specificity of EMSA, probes of functional inducible promoter sequence (−74 to +67), inactive promoter sequence (−55 to +67, containing half of the *cis*-element) and promoter sequence of −26 to +67 (lacking the *cis*-element) were used. As shown in Fig. 1A, only the nuclear extract from 3T3-L1 adipocytes caused significant DNA-protein interaction in EMSA. Little protein–DNA interaction was observed with 3T3-L1 preadipocyte nuclear extract. Several *trans*-protein-probe complexes were found with labeled −74 sequence probe and adipocyte nuclear extract. The EMSA pattern of −55 sequence probe significantly differed from that of −74 sequence probe (Fig. 1A). It had only 3 strong protein–probe complexes, which were also produced by −74 probe, but lacked those slow-moving protein-probe complexes found with −74 probe. −26 to +67 sequence probe produced no protein-DNA complex at all. These EMSA results were in consistence with the results of promoter functional analysis and DNase I footprinting (data published in another paper). They also confirmed the observation from DNase I footprinting that only nuclear extract of adipocyte bound to this DNA region.

To ascertain the specificity of these *trans*-proteins, competition experiment was conducted (Fig. 1B). The proteins interacted with −55 sequence probe could be competed off by excess unlabeled −55 probe or −74 probe. However, proteins bound to −74 probe could only be competed off by unlabeled −74 sequence, not by −55 probe. Since DNA sequence from −65 to −46 is the *cis*-element, P1 oligonucleotide corresponding to this sequence was used in competition experiment with −74 probe. As shown in Fig. 1B, unlabeled P1 oligonucleotide competitor was just as effective as unlabeled −74 probe. Almost all the proteins bound to −74 probe could be competed off by P1 oligonucleotide.

EMSA results suggested that the *trans*-proteins bound to the DNA promoter region were differentially induced, as nuclear extract of preadipocytes did not bind to the promoter (Fig. 1A). Investigations were made to determine the differentiation stage at which these *trans*-proteins were activated or induced. EMSA using promoter sequence probe (−74 to +67) and nuclear extracts prepared from 3T3-L1 cells at various differentiation stages showed that *trans*-proteins bound to DNA probe were activated or induced immediately after the hormonal stimulation for 3T3-L1 preadipocyte differentiation (Fig. 1C). Nuclear extracts from induced 3T3-L1 cells (from day 1 to day 6) all produced similar protein–probe interacted complexes, while nuclear extract from noninduced cells (day 0) did not bind to the labeled probe. Since the DNA probe of −74 to +67 contained the putative TATA-box basic promoter, a DNA probe without the TATA-box basic promoter (−74 to −26) was prepared for EMSA. The

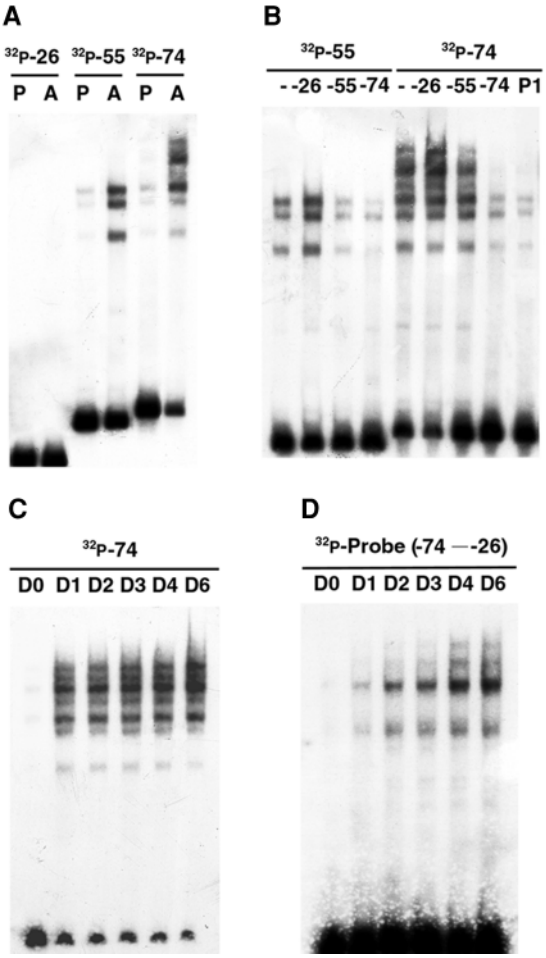

FIG. 1. Electrophoretic mobility shift assay (EMSA) of proteins bound to AAAT promoter. (A) EMSA with labeled AAAT promoter probes. (^{32}P-26) refers to ^{32}P labeled promoter DNA fragment from −26 to +67, (^{32}P-55) refers to DNA from −55 to +67 and (^{32}P-74) refers to DNA from −74 to +67. (P) indicates the nuclear extract prepared from 3T3-L1 preadipocytes (day 0 cells without induction) and (A) indicates the nuclear extract from 3T3-L1 adipocytes (day 6 cells after differentiation induction). (B) EMSA competition experiment using 50-fold excess of unlabeled DNA probes. (^{32}P-55) and (^{32}P-74) refer to the labeled DNA probes, which are the same as in A. Nuclear extract was from 3T3-L1 adipocytes. (−) indicates no competitor DNA was added. (−26), (−55) and (−74) indicate the competitor DNA fragments, which were −26 to +67, −55 to +67 and −74 to +67. (P1) indicates P1 oligonucleotide added as competitor (referring "Experimental Procedures" for sequence). (C) EMSA with nuclear extracts prepared from 3T3-L1 cells at indicated differentiation stages. (^{32}P-74) is DNA probe. (D0) to (D6) refer to nuclear extracts prepared from day 0 noninduced 3T3-L1 preadipocytes (D0) to 3T3-L1 adipocytes 6 days after differentiation induction (D6). (D) The same EMSA as in (C) except that DNA probe is shorter. It contained only the Ps1 site, from position −74 to −26.

result presented in Fig. 1D indicated that without the putative TATA box, DNA probe bound fewer *trans*-proteins. Nevertheless, these *trans*-proteins were also differentially induced. These results suggested that the activation or induction of these *trans*-activators occurred in the early differentiation induction stage.

Identification of transcription factors bound to the inducible cis-element as Sp1 and Sp3. The sequence of the inducible *cis*-element P1 contains a potential Nkx-2.5 binding site and a potential Sp1 binding site. CAT assay indicated that Nkx-2.5-like sequence in the promoter was not functionally critical (data published in another paper); nevertheless, the possibility of Nkx-2.5 as the transcription factor was not completely ruled out. Labeled oligonucleotide of P1, produced mobility-shifted complexes in EMSA, while labeled Nkx-2.5 oligonucleotide (20) and 11-bp probe, 5′-TTTCAA-GTCCC-3′ containing only the Nkx-2.5 like sequence of the *cis*-element, did not formed any protein-probe complex. This was confirmed by competition assay, as proteins bound to P1 probe could not be competed off by either unlabeled Nkx-2.5 or 11-bp probe (results not shown). In addition, Nkx-2.5 mRNA was not detected in 3T3-L1 adipocyte or mouse adipose tissue by Northern blot using Nkx-2.5 cDNA probe or RT-PCR with a pair of primers from mouse NKx-2.5 sequence (results not shown). Thus, transcription factor Nkx-2.5 could be ruled out.

Since Sp1-like sequence, 5′-GTCCCGCCCT-3′, in the *cis*-element is functionally important as mutations in this region completely inactivated the inducible promoter function, proteins bound to P1 probe and Sp1 probe were compared. Results shown in Fig. 2A indicated that both labeled P1 and Sp1 probes produced the same mobility-shifted complexes in EMSA; and they were each other's competitor. As transcription factor Sp1 is first identified in HeLa cells nuclear extracts from HeLa cells and 3T3-L1 adipocytes were compared for their binding to P1 and Sp1 probes. Again, both P1 and Sp1 probes produced the same protein-probe complexes in EMSA with HeLa cell nuclear extract (Fig. 2B). These results suggested that Sp1 is one of the transcription factors bound to P1 sequence. Since Sp3, another Sp1 family transcription factor, also recognizes Sp1-binding sequence, it is possible that Sp1 and Sp3 both bind to P1 sequence in 3T3-L1 adipocytes. Using EMSA supershift with antibodies against Sp1 and Sp3, it was clear that both Sp1 and Sp3 recognized and bound to this *cis*-element in AAAT promoter. Of the three major protein-P1 complexes in EMSA, two were by transcription factor Sp3 and the upper most complex band was by Sp1 (Figs. 3A and 3B).

CAT expression analysis with mutated AAAT promoter showed that mutations in Sp1-like core region inactivated the inducible promoter activity, while mutation in Nkx-2.5-like region did not affect the promoter function (data published in another paper). Thus, their ability to affect the binding of Sp1 and Sp3 to the promoter element was analyzed (Fig. 2C). The results showed that unlabeled Pm1 and Pm2 probes, corresponding to the mutation in Sp1-like sequence (see Materials and Methods for sequences) were unable

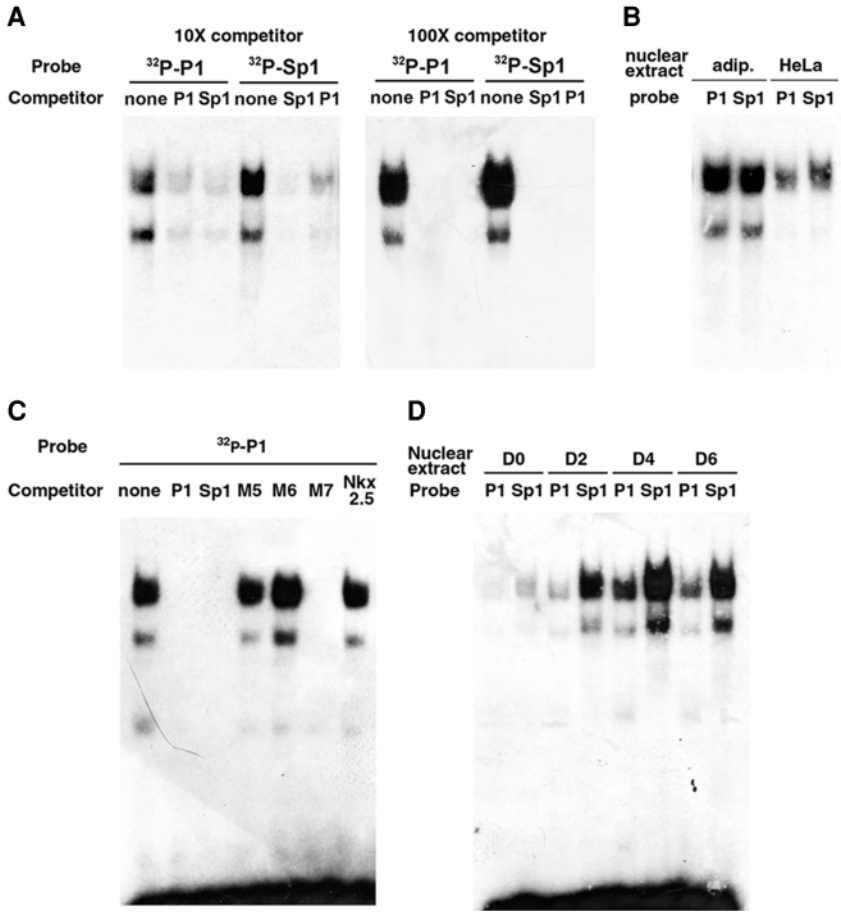

FIG. 2. Identification of transcription factor binding to AAAT promoter inducible cis-element as Sp1 family transcription factors. (A) ^{32}P-labeled P1 oligonucleotide and Sp1 binding oligonucleotide were used to conduct EMSA and competition analysis. Nuclear extract was prepared from differentiated 3T3-L1 adipocytes. (B) Comparison of EMSA using 3T3-L1 adipocyte nuclear extract and HeLa cell nuclear extract. The probes were labeled P1 and Sp1 oligonucleotides. (C) EMSA competition experiment. Labeled P1 oligonucleotide and 3T3-L1 adipocyte nuclear extract were used in EMSA. 100-fold excess unlabeled oligonucleotides indicated in the figure were added in the experiment. M5, M6 and M7 are mutated P1 oligonucleotide corresponding to the mutations for CAT assay (Fig. 3C and Materials and Methods for sequences). (D) Binding of transcription factor Sp1 to P1 and Sp1 oligonucleotides during 3T3-L1 cell differentiation process. (D0) nuclear extract prepared from noninduced 3T3-L1 preadipocytes; (D2) nuclear extract prepared from 3T3-L1 cells two-day after differentiation induction; (D4) and (D6) nuclear extracts prepared from day 4 cells and day 6 differentiated 3T3-L1 adipocytes.

to compete for P1 oligonucleotide probe, while unlabeled Pm3, corresponding to the mutation in Nkx-2.5-like sequence, completely competed off Sp1 and Sp3 from P1 probe. This EMSA result is entirely consistent with the results of promoter functional analysis with CAT reporter gene.

Differentiation-activated binding of Sp1 and Sp3 to P1 in AAAT promoter. Northern analysis of AAAT mRNA indicates that its expression is activated during 3T3-L1 cell differentiation induction and is adipocyte specific (16). Results from promoter analysis supported that AAAT promoter activity and the binding of *trans*-activators was induced during 3T3-L1 cell differentiation induction. However, Sp1 and Sp3 are ubiquitous transcription factors. Thus, possibilities are considered that Sp1 and Sp3 undergo an activation process during 3T3-L1 cell differentiation process. Consistent with the previous results, binding of transcription factors Sp1 and Sp3 to P1 probe were differentially induced as shown in Fig. 2D. To determine the change of Sp1 and Sp3 protein amount during differentiation process, Western immunoblotting analysis was conducted. The result indicated that Sp1 protein existed at high level in both noninduced and induced 3T3-L1 cells (Fig. 4A). Although Sp3 was markedly increased in differentiated cells than noninduced cells, there was still significant amount of Sp3 protein in noninduced 3T3-L1 preadipocytes (Fig. 4B). Thus, it was unlikely that the increased binding of Sp1 and Sp3 to AAAT promoter was due to increased protein level of Sp1 and Sp3.

Involvement of dephosphorylation in Sp1 activation during differentiation induction. Since Sp1 and Sp3 proteins existed at high level in noninduced 3T3-L1 preadipocytes, yet they did not bind to AAAT promoter

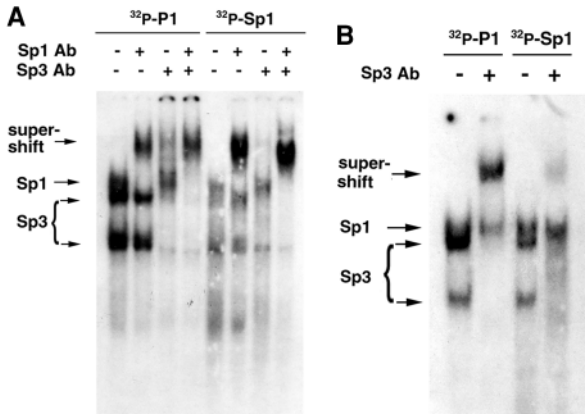

FIG. 3. EMSA supershift with anti-Sp1 and anti-Sp3 antibodies. (A) DNA probes were labeled P1 and Sp1 oligonucleotides and nuclear extract was prepared from 3T3-L1 adipocytes. 0.1 μg of Sp1 antibody and/or 0.1 μg Sp3 antibody were added in EMSA. (B) One microgram of Sp3 antibody was added in EMSA, and the rest was same as in A.

in high affinity. Phosphorylation or dephosphorylation of Sp1 and/or Sp3 was considered to be involved in their activation. After treatment with calf intestinal alkaline phosphatase, nuclear extract of noninduced 3T3-L1 preadipocytes (day 0 cells) exhibited markedly increased P1-binding activity, especially for Sp1 protein (Fig. 5A). The binding of Sp3 to labeled P1 probe was also increased after the phosphatase treatment, but to a less extend than Sp1. Thus, a dephosphorylation step could be involved in the activation of Sp1 during differentiation induction process. Western blot analysis confirmed this observation. As shown in Fig. 5B, after dephosphorylation by calf intestinal alkaline phosphatase, Sp1 protein changed from double-band to a single-band. Thus, the upper Sp1 protein band is

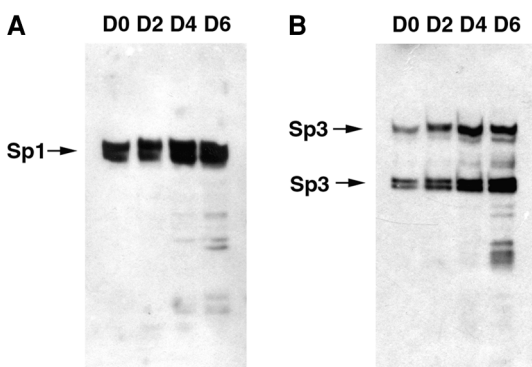

FIG. 4. Western immunoblotting analysis of Sp1 and Sp3 proteins in 3T3-L1 cell differentiation process. Nuclear extracts from 3T3-L1 cell at several differentiation stages were subjected to SDS–PAGE and transferred to membrane. After immunoblotting with anti-Sp1 antibody (A) or anti-Sp3 antibody (B), the proteins were visualized with ECL. The nuclear extracts are the same as used in Fig. 6D.

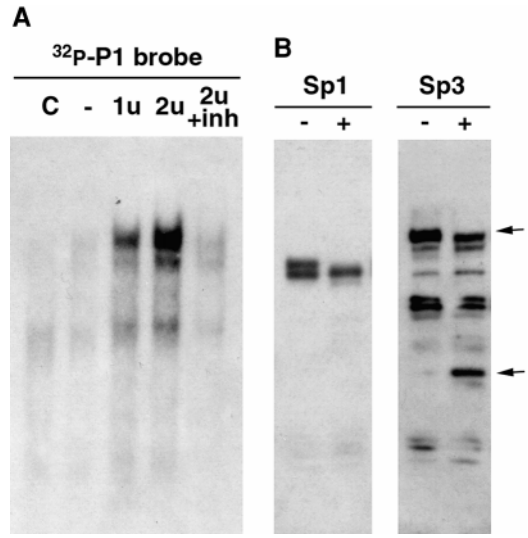

FIG. 5. Activation of Sp1 and Sp3 by dephosphorylation. (A) EMSA with nuclear extracts treated with phosphatase. Day 0 3T3-L1 preadipocyte nuclear extract was used in dephosphorylation reaction. ^{32}P-labeled P1 oligonucleotide was used as the DNA probe. (C) nuclear extract without treatment. (−) nuclear extract incubated as in dephosphorylation reaction but without the addition of phosphatase. (1u) nuclear extract treated with one unit of calf intestinal alkaline phosphatase. (2u) nuclear extract treated with two units of calf intestinal alkaline phosphatase. (2u + inh) nuclear extract treated with two units of calf intestinal alkaline phosphatase in the presence of 1 mM sodium vanadate and 30 mM β-glycerol phosphate. (B) Western blots of nuclear extract with or without phosphatase treatment. Noninduced day 0 preadipocyte nuclear extract treated with (+) or without (−) 2 units of calf intestinal alkaline phosphatase was subjected to Western immunoblotting with anti-Sp1 antibody (Sp1) or anti-Sp3 antibody (Sp3). The proteins were visualized with ECL.

likely to be the phosphorylated form. The change of Sp3 protein was not as significant as Sp1 protein, but there might be some minor changes as indicated in the figure.

DISCUSSION

Most genes induced during adipocyte differentiation process, such as *422/aP2*, *Glut4*, *SDC1*, *OB*, etc. are trans-activated by C/EBPα and/or PPARγ (21–25), which are two most important transcription factors in adipocyte differentiation regulation. They have a common feature that there is C/EBPα and/or PPARγ binding DNA motif in their promoters. However, AAAT gene, an adipocyte differentially induced gene (16), did not contain a functionally important C/EBPα or PPARγ binding motif in its promoter, rather it had a functionally important Sp1-like motif, which is responsible for its adipocyte differentiation-induced expression (data published in another paper). This implicated Sp1 family transcription factors in the transcriptional regulation of some of the adipocyte-specific genes. In the present

study, we demonstrated that transcription factors Sp1 and Sp3 were the *trans*-activators bound to this inducible *cis*-motif in AAAT promoter. Promoter functional analysis with CAT activity expression suggests that binding of Sp1-like *cis*-motif by Sp1 and Sp3 is only sufficient for activating the promoter during early differentiation induction. In differentiated adipocyte, Sp1 and Sp3 still bind to the promoter (Fig. 2), but they are not able to activate the promoter. It is possible that some modification on the Sp1 or Sp3 may inactivate their function in adipocyte. Thus, Sp1 and/or Sp3 are activated during 3T3-L1 preadipocyte differentiation induction, then bind and activate AAAT promoter. In late differentiation stage, their binding to the promoter is still essential, but is not functionally sufficient. Some other transcription factors may be required for activating AAAT promoter in terminally differentiated adipocyte.

Since high level of Sp1 and Sp3 proteins exists in 3T3-L1 preadipocyte, increased binding of Sp1 and Sp3 to AAAT promoter after induction is likely due to protein activation. Our result indicates that the activation of Sp1 might involve a dephosphorylation step, as treatment of noninduced 3T3-L1 preadipocytes nuclear extract with calf intestinal alkaline phosphatase significantly increased the binding affinity of Sp1 to P1 *cis*-element (Fig. 5). It is not clear the phosphorylation state of Sp1 in preadipocyte. The phosphatase treatment reduced Sp1 to a single band that seems to exist before the treatment. Multiple phosphorylation may be one of the reasons that Sp1 did not bind to DNA with high affinity before the phosphatase treatment. Many studies have indicated that phosphorylation and dephosphorylation of Sp1 regulate its DNA binding activity (15, 26–31). Depending upon systems, phosphorylation and dephosphorylation of Sp1 may play a different role. In general, the phosphorylation state of Sp1 is an important regulatory modification for Sp1 activity. It has been shown that regulation of PTPase activity during 3T3-L1 preadipocyte differentiation induction is important for the cell differentiation (32). Thus, the activation of Sp1 by dephosphorylation during differentiation induction might be related to this PTPase activity regulation.

The role of Sp3 in AAAT gene differential expression is less clear. In our study, the binding of Sp3 are always accompanied by Sp1 vice versa, and they both were activated after the differentiation induction. It is possible that Sp1 and Sp3 act synergistically to activate AAAT promoter during differentiation induction. Combining with our previous studies, it is clear that transcription factors Sp1 and Sp3 binding to the Sp1-like *cis*-element activates AAAT gene expression during 3T3-L1 cell differentiation induction. Thus, it is possible that Sp1 family transcription factors play some roles in regulating adipocyte differentiation.

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