Nutritional and Insulin Regulation of Fatty Acid Synthetase and Leptin Gene Expression through ADD1/SREBP1

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Abstract

The ability to regulate specific genes of energy metabolism in response to fasting and feeding is an important adaptation allowing survival of intermittent food supplies. However, little is known about transcription factors involved in such responses in higher organisms. We show here that gene expression in adipose tissue for adipocyte determination differentiation dependent factor (ADD) 1/sterol regulatory element binding protein (SREBP) 1, a basic-helix-loop-helix protein that has a dual DNA-binding specificity, is reduced dramatically upon fasting and elevated upon refeeding; this parallels closely the regulation of two adipose cell genes that are crucial in energy homeostasis, fatty acid synthetase (FAS) and leptin. This elevation of ADD1/SREBP1, leptin, and FAS that is induced by feeding in vivo is mimicked by exposure of cultured adipocytes to insulin, the classic hormone of the fed state. We also show that the promoters for both leptin and FAS are transactivated by ADD1/SREBP1. A mutation in the basic domain of ADD1/SREBP1 that allows E-box binding but destroys sterol regulatory element-1 binding prevents leptin gene transactivation but has no effect on the increase in FAS promoter function. Molecular dissection of the FAS promoter shows that most if not all of this action of ADD1/SREBP1 is through an E-box motif at −64 to −59, contained with a sequence identified previously as the major insulin response element of this gene. These results indicate that ADD1/SREBP1 is a key transcription factor linking changes in nutritional status and insulin levels to the expression of certain genes that regulate systemic energy metabolism. (*J. Clin. Invest.* 1998. 101:1–9.)

Key words: ADD1/SREBP1 • fatty acid synthetase • leptin • nutritional changes • insulin

Introduction

Mammals and other higher species have evolved complex mechanisms to maintain a constant supply of metabolic energy in the face of highly variable food supplies. Adipose tissue is the primary site of energy storage, building triglycerides in times of nutritional excess and releasing this energy in the form of FFA and glycerol in times of fasting. The ability to carry out these functions efficiently is dependent on changes in the expression of genes that carry out the lipogenic or lipolytic programs. The adipose depot is not an entirely passive player in this energy homeostasis scheme; adipose cells can secrete molecules that limit obesity through effects on food intake and energy expenditure (leptin; reference 1) and control insulin sensitivity of other tissues (TNF-α; references 2 and 3).

Despite the fact that changes in gene expression are an important component in the response of adipose and other tissues to altered nutritional states, the precise mechanisms responsible for this are not known. Several response elements at the DNA level have been described for glucose or insulin-dependent regulation (4, 5), but no transcription factors playing well-defined roles have been identified. One interesting candidate could be adipocyte determination differentiation dependent factor (ADD) 1/sterol regulatory element binding protein (SREBP)[1] 1. This transcription factor, cloned originally from rat adipose tissue (6), has been shown to regulate the expression of several key genes of fatty acid and triglyceride metabolism in cultured fibroblasts, adipocytes, and the livers of transgenic mice (7–9). Fatty acid synthetase (FAS), in particular, has been shown to be regulatable by ADD1/SREBP1 (7, 8, 10) and sensitive to nutritional perturbations in vivo (11, 12). The human homolog of ADD1 (SREBP1) and a closely related factor (SREBP2) have been cloned independently and shown to be associated with the regulation of several genes controlling cholesterol homeostasis (13, 14). Notably, depletion of cholesterol causes proteolytic release of the transcriptionally active amino-terminal portion of these molecules from their position embedded in the endoplasmic reticulum membrane to allow movement to the nucleus (15, 16). Based on a range of data generated in vivo and in cultured cells, it is likely that SREBP2 is associated more specifically

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1. *Abbreviations used in this paper:* ADD, adipocyte determination differentiation dependent factor; bHLH, basic-helix-loop-helix; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer–binding protein; EMSA, electrophoretic mobility shift assay; FAS, fatty acid synthetase; LPL, lipoprotein lipase; SRE, sterol regulatory element; SREBP, SRE-binding protein.

with cholesterol metabolism, while ADD1/SREBP1 plays a major role in the control of genes involved in metabolism of fatty acids and other lipids (7, 8, 17, 18).

ADD1/SREBP1 is very unusual in that it has a dual DNA-binding specificity (19). Like most members of the basic-helix-loop-helix (bHLH) family, it can bind to E-box sequences having a consensus CANNTG (20). However, unlike any other member of this family, it also binds the sterol regulatory element (SRE)-1 sequence (ATCACCCCAC), first associated with genes of cholesterol metabolism (21, 22). This dual DNA-binding specificity of ADD1/SREBP1 is due entirely to an atypical tyrosine in the basic DNA-binding domain, where all other bHLH factors have an arginine (19). These DNA-binding characteristics are noteworthy because several (but not all) identified insulin response elements appear to be E-boxes, including one in the FAS promoter (4, 23).

In this study, we show that the expression of ADD1/SREBP1 is altered dramatically during fasting and refeeding of mice, closely paralleling the regulation of two key genes of energy homeostasis, FAS and leptin. ADD1/SREBP1 transactivates both of these promoters, regulating FAS primarily through its identified insulin response element. These data identify ADD1/SREBP1 as an important transcriptional regulator of adipose cell genes sensitive to nutritional status and insulin.

Methods

Animal treatment. Female C57BL/6 mice (10–12 wk, 18–22 g) were housed (five mice/cage) and raised on open formula rat/mouse rations (Zeigler Brothers, Inc., Gardners, PA), and water was given ad libitum, with a 12-h light–dark cycle beginning at 6:30 a.m. All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health. In experiments, food was withdrawn during the daylight hours before onset of the dark cycle. In the refeeding experiment, food was reintroduced after 7.5 h of fasting. Animals were killed at indicated time points for the isolation of epididymal fat.

Northern blot analysis and RNase protection assay. Total RNA was isolated from the epididymal adipose tissue of mice and cultured cells by a guanidine isothiocyanate extraction protocol described previously (6, 7). 10–30 μg of RNA was denatured in formamide and formaldehyde, and subsequently separated by electrophoresis in formaldehyde-containing agarose gels. RNA was transferred to nylon membrane (ICN Biomedicals, Inc., Costa Mesa, CA), and membranes were cross-linked, hybridized, and washed as described by the manufacturer. DNA probes were labeled with [α-^{32}P]dCTP (6,000 Ci/mmol) with a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cDNA containing the sequence corresponding to murine SREBP1a mRNA (−102 to +200; nucleotide 1 is the A of the ATG codon that encodes the first methionine) was cloned by PCR. The amplified cDNA was subcloned into pGEM-7Zf(+) (Promega Corp., Madison, WI). After linearization of plasmid DNA, antisense RNA was transcribed with [α-^{32}P]UTP using T7 RNA polymerase (Ambion Inc., Austin, TX). RNase protection assays were performed with an RPA II kit (Ambion Inc.) as recommended by the manufacturer. Briefly, sample RNA (5 or 10 μg) was mixed with the ^{32}P-labeled riboprobe in a hybridization buffer, and the mixtures were denatured and hybridized. After digestion with RNase A/T1, protected fragments were separated on a 6% polyacrylamide gel containing 8 M urea. Gels were dried and then subjected to autoradiography.

Cell culture. 3T3-L1 preadipocytes were grown in DME containing 10% bovine calf serum. Differentiation of 3T3-L1 cells was induced as described previously (24). At confluence, the medium was changed to DME supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethyl xanthine, and 5 μg/ml of insulin for 2 d. Thereafter, the medium was replaced every other day with 5 μg/ml of insulin and 10% FBS. Rat1-IR cells were grown in DME/F12 (50:50) medium containing 10% FBS as described previously (25).

Transient transfections and enzyme assays. Rat1-IR cells were cultured as described above and transfected 1 d before confluence by the calcium phosphate method described previously (25). To examine insulin effects on the FAS promoter, transfected Rat1-IR cells were incubated in 0.5% BSA (ICN Biomedicals, Inc.) overnight. The following day, these serum-deprived cells were treated with insulin (100 nM) for 24 h. Cells were then harvested for enzyme assays. All the mammalian expression vectors for ADD1-403, ADD1-403R, and CCAAT-enhancer–binding protein (C/EBP)α are derived from pSV-SPORT1 (GIBCO BRL, Gaithersburg, MD). Each 60-mm-diameter dish received either 1 μg of luciferase reporter plasmid with rat FAS promoter (−220 to +25) or chloramphenicol acetyltransferase (CAT) reporter plasmid with mouse leptin promoter (−6.5 kb to +9) (GenBank accession number U65742). In addition, each dish received 0.5 μg of pCH110 plasmid containing the lacZ gene (Pharmacia Biotech) and either ADD1 (1 μg) or C/EBPα (1 μg) expression vectors. The level of CAT expression was determined by measuring CAT enzyme activity as described previously (19). Luciferase activity was measured in a luminometer with a luciferin reagent (Boehringer Mannheim Biochemicals). β-Galactosidase assays were performed by a standard colorimetric procedure with chlorophenol red–β-D-galactopyranoside as substrate (Boehringer Mannheim Biochemicals). The relative CAT and luciferase activities were normalized to the β-galactosidase activity. All the transfection experiments were performed in duplicate and repeated at least three times independently.

Electrophoretic mobility shift assay and plasmids. EMSAs were performed as described (19). In vitro–translated ADD1-403 and ADD1-403R proteins were produced using the TNT SP6-coupled reticulocyte lysate system (Promega Corp.). For binding assays, reactions were carried out in 20-μl volume containing in vitro–translated protein lysate (1–5 μl) and ^{32}P-labeled probe (0.1 pmol). DNA–protein complexes were resolved on a 5% polyacrylamide gel. The gel was dried and exposed to film at −70°C. The FAS promoter mutations described in the citations to Fig. 6 were generated by ligation of three synthetic oligonucleotides. The DNA sequence of the wild-type and mutant FAS promoter constructs was confirmed by DNA sequencing analysis, and subsequently cloned into a plasmid containing a luciferase reporter gene.

Results

Nutritional and insulin regulation of ADD1/SREBP1 gene expression. A uniform, synchronized feeding state was established by removing food during the daylight hours, when mice usually do not feed extensively. At the start of the night cycle (6:30 p.m.), animals were either provided with food or deprived of food for 7.5 or 12 h. A fourth group of mice was refed for 4.5 h after the 7.5-h fast. As can be seen in Fig. 1, *A* and *B*, ADD1, leptin, and FAS mRNA expression decreased dramatically after the 7.5- or 12-h fast, and refeeding rapidly restored these to a level equal to or exceeding the original fed state. The maximal decrease was 90% for ADD1 and leptin and 75% for FAS. In contrast, mRNA for other important fat cell transcriptional regulators such as peroxisome proliferator–activated receptor γ and C/EBPα showed little change during this relatively short-term fast. Lipoprotein lipase (LPL) mRNA also underwent little alteration during these treatments. As illustrated in Fig. 1 *B*, the decreases in ADD1, leptin, and FAS are similar in relative magnitude and time course, suggesting parallel regulation. In data not shown, we have found that sim-

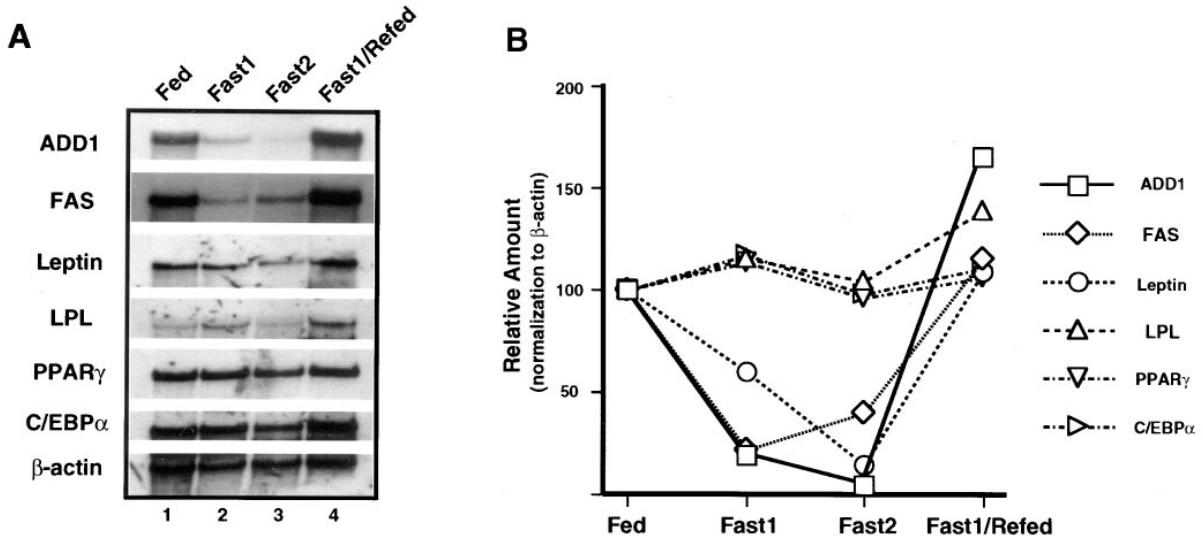


Figure 1. Nutritional regulation of ADD1, FAS, and leptin gene expression. (*A*) mRNA expression levels in adipose tissue from fed and fasted mice were compared by Northern blot analysis. Mice were killed at different time points, and total RNA was prepared from epididymal adipose tissue. Mice were divided into four groups. A fed control (*Fed*) was allowed free access to food for 7.5 h (lane *1*). Both fasted groups (*Fast1* and *Fast2*) were denied access to food for 7.5 and 12 h, respectively (lanes *2* and *3*). The refed group (*Fast1*/*Refed*) was allowed free access to food for 4.5 h after 7.5 h of fasting (lane *4*). Equivalent amounts (10 μg) of loaded RNA were run in each lane. (*B*) Quantitation of feeding and fasting effects. All the expression levels were normalized to β-actin gene expression. Expression of each gene was plotted relative to the control fed mice.

ilar regulation of ADD1/SREBP1 mRNA by fasting and refeeding is seen in the liver.

The particular hormones or nutrients responsible for this response to fasting and feeding are of considerable interest. Since insulin is the classic hormone of the fed state, and FAS and leptin have been shown previously to be induced by insulin (26, 27), we asked whether this hormone controls ADD1 expression in a parallel fashion. Indeed, insulin treatment at 100 nM increases ADD1 expression after 2 h, and this continues to increase until 6 h (Fig. 2 *A*). Leptin and FAS mRNAs show similar increases, though as published previously, the levels of leptin mRNA are very low in these cultured fat cells (11, 26–28).

Since insulin can function through its own receptor or, at very high doses, through the IGF-1 receptor, an insulin dose–response experiment was performed at a single time point (24 h). As shown in Fig. 2 *B*, ADD1 expression is maximal at 10 nM and then decreases somewhat at higher doses. This mirrors closely the levels of FAS mRNA expression, while leptin expression appears relatively similar for doses between 2 and

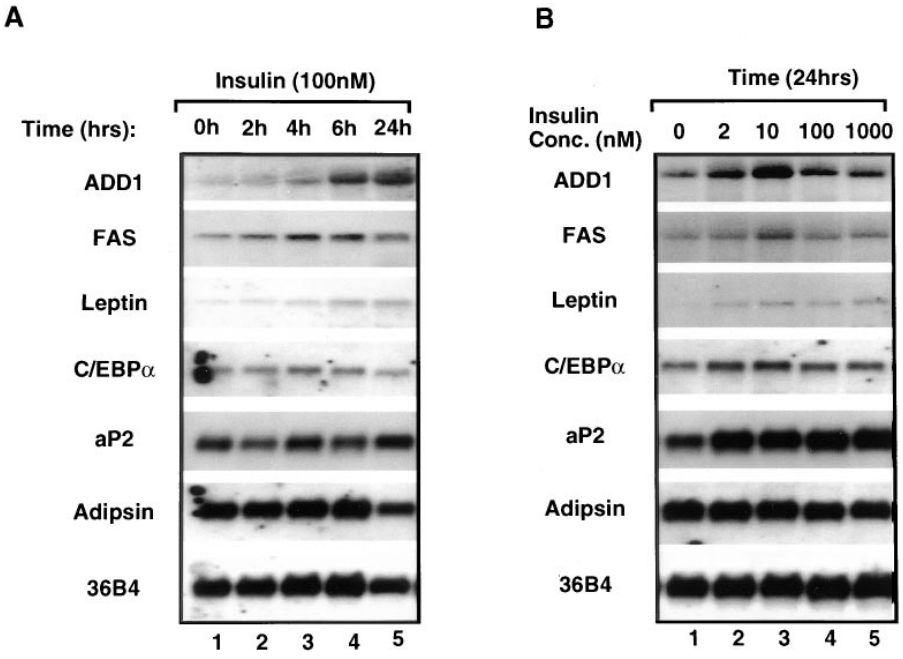


Figure 2. Insulin induction of ADD1 mRNA. (*A*) Alteration of gene expression induced by insulin treatment. Fully differentiated 3T3-L1 adipocytes were incubated for 2 d in serum-free low glucose DME containing 0.5% BSA, and were then treated with 100 nM insulin. Cells were harvested at various time points to prepare total RNA. (*B*) Dose–response of insulin-induced gene expression. Fully differentiated 3T3-L1 adipocytes were incubated for 2 d in serum-free low glucose DME with 0.5% BSA. Adipocytes were then treated with or without insulin at a concentration range of 2–1,000 nM for 24 h. Cells were harvested to prepare total RNA (30 μg) for Northern blot analysis. Equivalent amounts of loaded RNA were run in each lane, as indicated by hybridization to 36B4 probe.

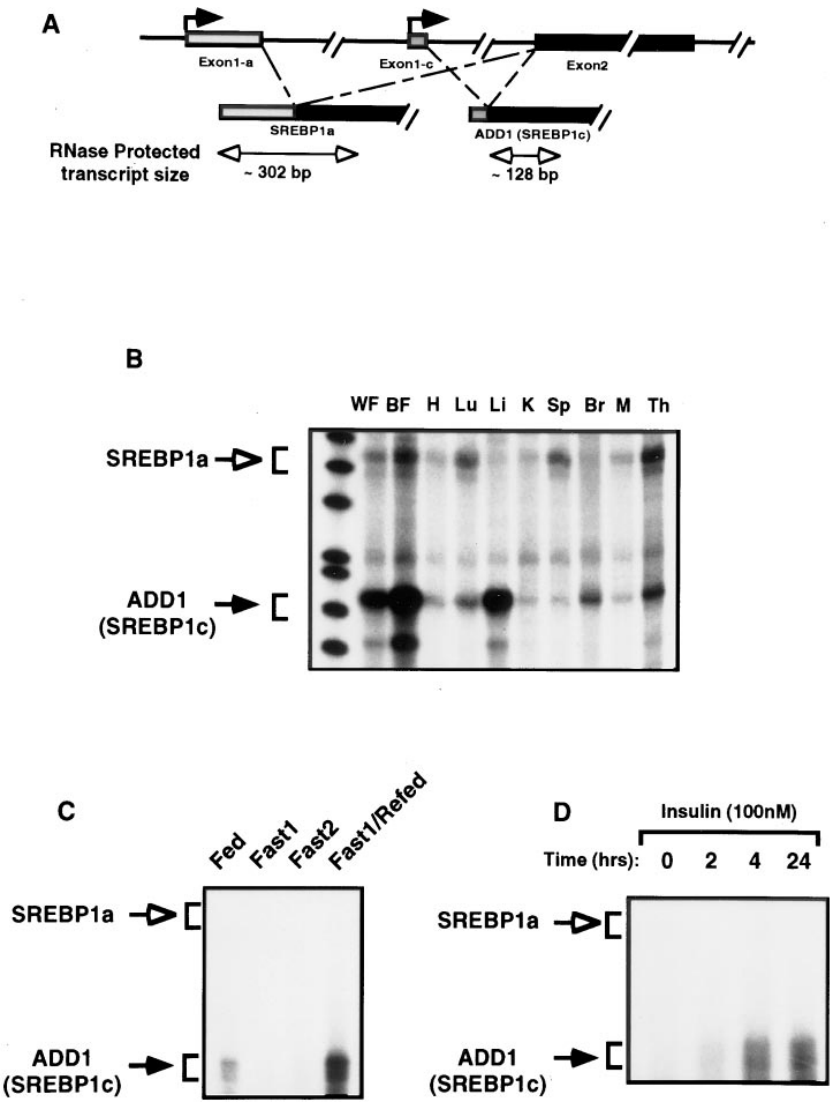


Figure 3. Nutrition and insulin regulation of ADD1/SREBP1 splicing variants. (*A*) Genomic structure of ADD1/SREBP1 gene. SREBP1a and ADD1/SREBP1c mRNAs are produced by alternate splicing of the first two exons in the ADD1/SREBP1 gene. RNase protection assays generate two different sizes ($\sim$ 320 and $\sim$ 128 bp) of SREBP1a and ADD1/SREBP1c transcripts, respectively. (*B*) Expression pattern of ADD1/SREBP1 transcripts from mouse tissues. *WF*, White fat. *BF*, Brown fat. *H*, Heart. *Lu*, Lung. *Li*, Liver. *K*, Kidney. *Sp*, Spleen. *Br*, Brain. *M*, Muscle. *Th*, Thymus. (*C*) Effects of fasting and refeeding on the ADD1/SREBP1 form in white fat. (*D*) Insulin regulation of ADD1/SREBP1 forms in cultured adipocytes. RNAs used in *C* and *D* were the same as those used in Fig. 1 *A* and Fig. 2 *A*, respectively. RNase protection assays were performed as described in Methods.

1,000 nM. The relatively low amount of insulin required for ADD1 expression is consistent with an action through the insulin receptor (29). The lack of exact parallel between expression of ADD1/SREBP1 and leptin suggests that other transcription factors besides or in addition to this one may be important in insulin regulation of the leptin gene.

ADD1/SREBP1 mRNA has been shown to exist in two forms generated by alternative splicing, differing in their 5′ termini. ADD1, equivalent to SREBP1c, has been shown to be the major form of mRNA expressed in adipose tissue in vivo (30). To investigate which form of ADD1/SREBP1 is regulated by nutritional status and insulin, we used an RNase protection assay, which can detect both splicing variants. In agreement with Shimano and colleagues (30), ADD1/SREBP1c is the major form of this gene expressed in white and brown fat, while SREBP1a represents a significant portion of total transcripts from lung, spleen, and thymus (Fig. 3 *B*). Fig. 3 *C* illustrates that the ADD1/SREBP1c form is also the major form regulated by fasting/refeeding in white adipose tissue. It appears to be the major splicing variant seen in cultured 3T3-L1 adipocytes, and is induced by insulin treatment of cultured adipocytes (Fig. 3 *D*). The very low level of SREBP1a expressed in fat tissue in vivo or in cultured adipocytes did not allow us to determine whether it too is regulated.

ADD1/SREBP1 transactivates the FAS and leptin genes. To investigate the potential of ADD1/SREBP1 to regulate the FAS and leptin genes, promoter fragments were obtained and linked to reporter genes luciferase or CAT. We used two alleles of ADD1 — one encoding the amino-terminal fragment of the wild-type protein that has maximal activity (ADD1-403), and a second that is similar except it contains a tyrosine to arginine mutation at position 320 amino acid (termed ADD1-403R). This mutation has been shown to disrupt the dual specificity of DNA binding, allowing unaltered binding to E-boxes but destroying completely binding to SRE motifs (19). Fig. 4 *A* shows that cotransfection of the wild-type 1–403 ADD1 allele with a −220 to +25–bp fragment of the FAS promoter results in a fivefold stimulation of activity. Interestingly, the ADD1-403R mutant allele does not reduce this transactivation at all, suggesting that the response element(s) in the FAS promoter is an E-box motif. The wild-type allele of ADD1 strongly transactivates the leptin promoter (Fig. 4 *B*)

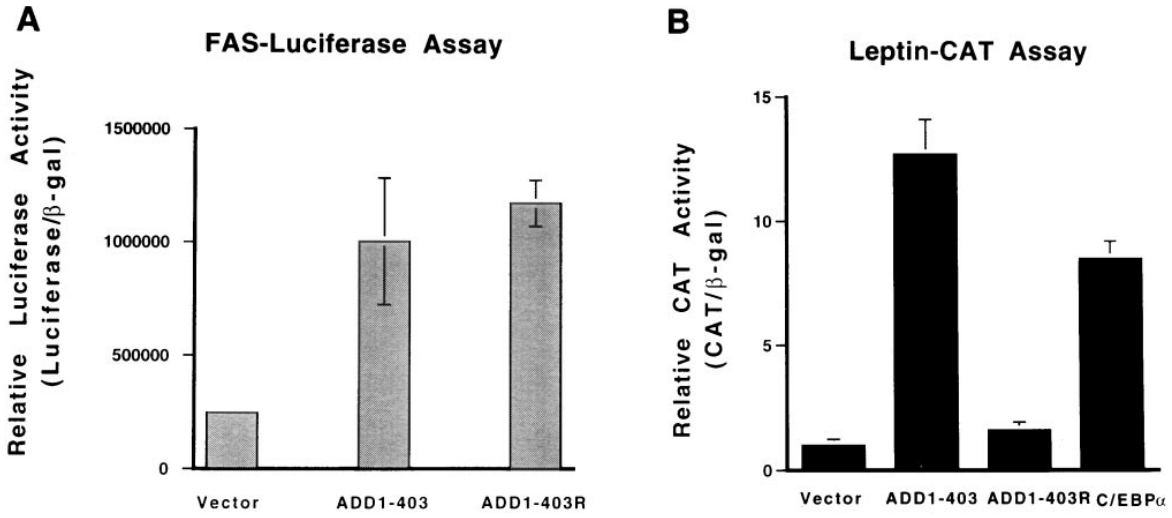
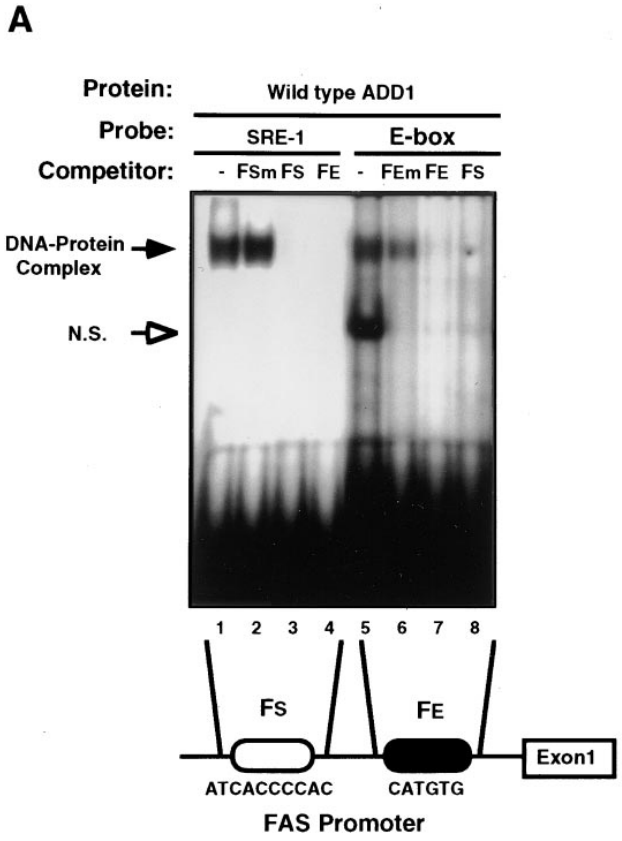


Figure 4. ADD1 transactivates the promoters of the FAS and leptin genes. (*A*) Ectopic expression of ADD1 activates the promoter of the FAS gene. Rat1-IR cells were cotransfected with FAS–luciferase reporter containing the FAS promoter (−220 to +25) and either wild-type ADD1 or ADD1-403 mutant expression vectors. Relative luciferase activity was calculated by normalization to β-galactosidase activity driven by pSV-SPORT1 control vector. (*B*) Ectopic expression of ADD1 activates the promoter of the leptin gene. Rat1-IR cells were cotransfected with the leptin–CAT construct containing the leptin promoter (−6.5 kb to +9) and several different expression vectors. Relative CAT activity was normalized to β-galactosidase.

by more than 12-fold. As has been shown previously, this promoter also responds to C/EBPα (31, 32). In contrast to the results with the FAS promoter, the Y→R mutation at position 320 essentially prevents completely the transactivation of the leptin gene by ADD1. This suggests that an E-box target is not sufficient for transactivation, most likely implicating an SRE-type element in the leptin promoter.

The ADD1/SREBP1 response element in FAS is an identified insulin response element E-box. We have studied the sites where ADD1/SREBP1 binds and functions on the FAS pro-



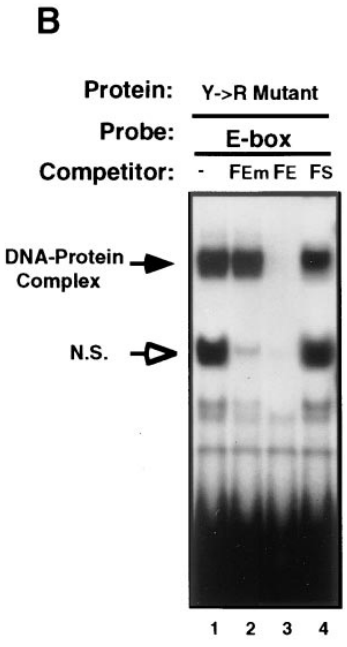


Figure 5. ADD1 binding sites in the FAS promoter. EMSA was performed with in vitro–translated ADD1-403 (*A*) or ADD1-403R (*B*) protein. The radiolabeled probes were prepared from oligonucleotides containing the SRE-1 sequence (*FS*, FAS–SRE probe) or the E-box sequence (*FE*, FAS–E-box probe). Among competitor oligonucleotides, *FS* indicates the SRE-1 motif of the FAS promoter, and *FE* indicates the oligonucleotide containing E-box motif of the FAS promoter. *FSm* and *FEm*, mutated *FS* and *FE* oligonucleotides, respectively. Competitor sequences are FS: gcgcggg-gATCA CCCCAC-cgacggc; FSm: gcgcgggAT-**TATCT**CACcgacggc; FE: ctgtcagccCATGTGgcgtg-gccg; FEm: ctgtcagc-c**TT**TGT**T**gcgtggccg. Mutated nucleotides are in bold. *N.S.*, Nonspecific DNA–protein complex.

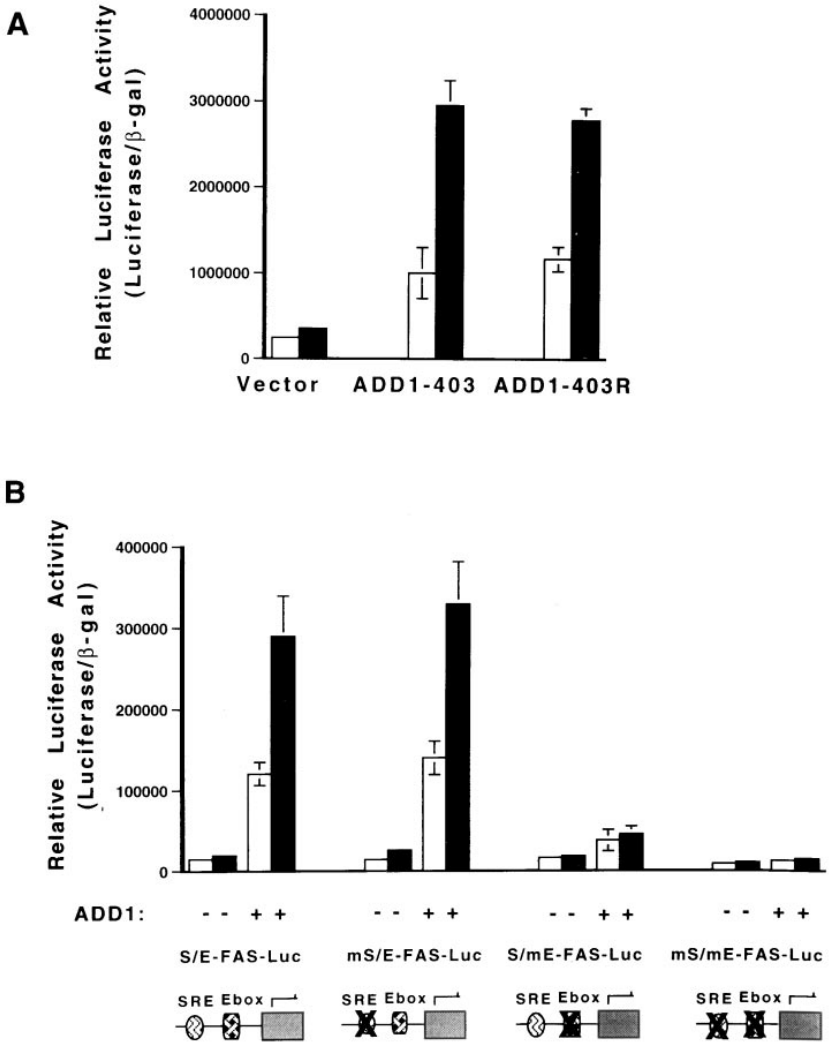


Figure 6. Mutational analysis of ADD1 action on the FAS promoter. (*A*) Rat1-IR cells were cotransfected with FAS–luciferase (*Luc*) construct containing FAS promoter, pCH110 β-galactosidase plasmid, and different ADD1 expression vectors. After transfections, cells were incubated overnight in serum-free DME with 0.5% BSA, and were then treated with (*black bars*) or without 100 nM insulin (*white bars*) for 24 h. Transfected cells were harvested and assayed for luciferase and β-galactosidase activity. (*B*) Various FAS–luciferase constructs containing mutations in either the SRE-1 or E-box were generated as described in Methods. Rat1-IR cells were cotransfected with each promoter fragment linked to luciferase with or without ADD1 expression vectors. After transfections, cells were treated as described above, and enzyme assays were performed. *S/E-FAS-Luc*, Reporter containing both wild-type SRE-1 and E-box motifs. *mS/E-FAS-Luc*, Reporter containing mutated SRE-1 and wild-type E-box motifs. *S/mE-FAS-Luc*, Reporter containing wild-type SRE-1 and mutated E-box motifs. *mS/mE-FAS-Luc*, Reporter containing mutations in both SRE-1 and E-box motifs. All the mutated sequences are described in the legend to Fig. 5.

moter. Visual and computer-assisted examination reveal two potential ADD1/SREBP1 binding sites, an SRE-1–type element at −150 to −141, and an E-box at −64 to −59. This E-box overlaps a sequence identified by Moustaid et al. as an insulin response element in the FAS promoter (10, 23). Fig. 5 *A* shows that in vitro–translated wild-type ADD1/SREBP1 binds to both of these sequences when they are presented as 24-mers in EMSA. We next investigated whether the Y→R mutation really distinguished E-box versus SRE binding at these promoters, as we have shown previously on sequences from other genes (19). Either oligonucleotide sequence (E-box or SRE-1) can compete specifically for binding of this protein to the cognate sequence or to the other binding sequence (Fig. 5 *A*). In contrast, the tyrosine to arginine mutation at residue 320 (ADD1-403R) can bind to the E-box oligonucleotide, but competition is only possible with the E-box sequence, not with a mutated E-box or the SRE element from the FAS gene (Fig. 5 *B*).

We next performed functional analysis of the FAS promoter that has the potential ADD1/SREBP1 binding sites disrupted sequentially or simultaneously by point mutations. In addition, because of the effects of insulin on the expression of endogenous ADD1/SREBP1 (Fig. 2), we have also used insulin treatments to ask whether there might be any further post-transcriptional effects of insulin on this protein. Fig. 6 *A* shows that insulin treatment for 24 h causes an approximately threefold further increase in the activity that ectopically expressed ADD1-403 elicits from the FAS promoter. The ADD1-403R mutant shows an identical effect.

Mutation first of the SRE sequence at −150 to −141 did not diminish the response to ADD1/SREBP1, with or without insulin (Fig. 6 *B*). In contrast, mutation of the E-box at position −64 to −59 had two effects: it decreased overall activity of the promoter by 80%, and it completely ablated the effect of added insulin (Fig. 6 *B*). A double mutation of the SRE and E-box renders this promoter completely resistant to activation by ADD1/SREBP1. These data indicate that most of the transactivation of this FAS promoter by ADD1/SREBP1 goes through the E-box motif at position −64 to −59. These data indicate a clear linkage between the nutrition and insulin regulation of ADD1/SREBP1 shown here and the E-box sequence identified as an insulin response element in the FAS promoter.

Discussion

The last 10 years have seen a vast increase in our knowledge of how nutrients and hormones controlled by diet can influence gene expression. The classic hormones of the fasted state, catecholamines and glucagon, function by raising cAMP levels, which can result in the activation of the transcription factor cAMP response element–binding protein through protein kinase A (33). This factor then functions in a variety of fasting-sensitive genes through a canonical cAMP response element–binding protein binding site. Fasting also leads to an elevation of glucocorticoids, which control gene expression mainly if not exclusively through the glucocorticoid receptor and its well-defined cognate sequence (34, 35).

The control of gene expression by feeding and the major feeding-associated hormone, insulin, have been more difficult to understand. Many insulin-sensitive genes have been studied, and there is little consensus as to what an insulin response element looks like (4). Even less is known about transcription factors that respond to insulin and regulate important genes of lipid and carbohydrate metabolism.

For a transcription factor to be described as an insulin response factor, several key criteria must be satisfied. First, it must participate in the regulation of the insulin-regulated target gene in question, as illustrated through ectopic expression (gain of function) or loss-of-function experiments in cells or whole animals. Second, the activity of the transcription factor itself must be regulated directly or indirectly by insulin. Finally, the target DNA sequence to which the gene control factor binds must be characterized. The work presented in this paper, in combination with previously published work from our laboratory and others (7, 8, 10, 23), establishes ADD1/SREBP1c as the first or among the first bona fide insulin response transcription factors.

Endogenous FAS expression has been shown previously to respond to changes in feeding status and insulin levels (11, 26). As shown in cultured cells and the livers of transgenic mice, ectopic expression of ADD1/SREBP1 alone is sufficient to increase greatly the expression of FAS and several other lipogenic genes (7, 8, 36). In cultured fibroblasts, the levels of ectopic ADD1/SREBP1 mRNA that can activate FAS expression are no greater than those normally expressed in cultured adipose cells or adipose tissue.

In the work presented here, insulin regulates ADD1/SREBP1 on at least two levels. It increases greatly its expression at the mRNA level and does so at doses of insulin consistent with a function through the insulin receptor. The dramatic regulation of ADD1/SREBP1 expression in adipose tissue observed during fasting and refeeding is consistent with this finding. Of course, it is entirely possible that other hormones or nutrients in addition to insulin participate in the regulation of ADD1/SREBP1 in this in vivo context. As shown in Fig. 6, ADD1/SREBP1 expressed by a viral promoter is also activated by the exposure of cells to insulin, suggesting an additional posttranscriptional activation. This has also been suggested by the experiments of Streicher et al., who examined the effects of insulin and IGF-1 in the LDL receptor gene (37).

Finally, the effects of ADD1/SREBP1 on the FAS promoter could be associated clearly with one particular target sequence, the E-box at −64 to −59. This is especially interesting because Sul and colleagues have identified a longer sequence that contains this element as the response element of FAS to insulin, in their studies performed in cultured adipocytes (23). When these various studies are combined, it is clear that ADD1/SREBP1 is functioning as an insulin response factor, at least in adipose tissue. Of course, further determination of the relative importance of this factor in the regulation of FAS, leptin, and other adipose-cell genes must await loss-of-function experiments involving genetic ablation of ADD1/SREBP1 in various tissues. Although our studies have focused primarily on fat tissue, the high levels of expression of ADD1/SREBP1 in liver suggest that this molecule could also be involved in the control of certain insulin-responsive genes in this tissue. On the other hand, Wang and Sul (in 1996) have suggested that upstream stimulatory factor 1, another member of the bHLH family, may be involved at the same key E-box control element in the liver (38). To date, there is no data indicating that ectopic expression of this factor can regulate FAS expression. Further studies will be required to clarify this point.

Despite the evidence that ADD1/SREBP1 can bind well to E-box sequences, it has been suggested recently that all or nearly all of the responses to the SREBPs were through SRE-type elements (39). Several lines of evidence now argue strongly for a function of ADD1 in the FAS promoter through the E-box. First, a mutant of ADD1/SREBP1 which can bind to E-boxes but not to SREs can transactivate the FAS promoter as well as the wild-type allele (Figs. 4 and 5). Second, specific mutations of the FAS promoter show that most if not all activity goes through the E-box at position −64 to −59 (Fig. 6). Finally, we have found most recently that the Y→R mutation of ADD1/SREBP1, targeted only to E-boxes, activates FAS gene expression effectively when ectopically expressed in fibroblasts (data not shown). Hence, the E-box motif must be considered the primary target of ADD1/SREBP1, at least for the FAS gene.

Feeding status has two important effects on adipose cells: it controls the amount of lipid in each cell, and can also affect the number of adipose cells. Most obesity generally involves an increase in both cell size and cell number. The data presented here and in earlier reports suggest that ADD1/SREBP1 may represent a plausible mechanism unifying both of these aspects of adipose cell biology (Fig. 7). While this factor alone is not sufficient to drive adipogenesis efficiently in fibroblasts, it can increase the percentage of cells that differentiate into adipocytes in response to a cocktail of inducing hormones (7). It appears to do this, at least in part, by increasing the transcriptional activity elicited from the adipogenic nuclear receptor

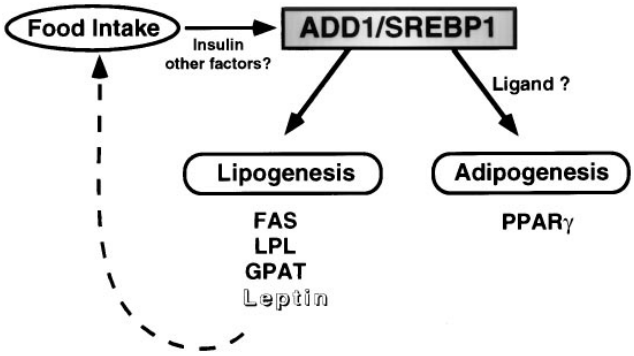


Figure 7. Role of ADD1/SREBP1 in lipogenesis and adipogenesis.

peroxisome proliferator–activated receptor γ, perhaps through the generation of an endogenous ligand. Hence, chronic overfeeding and its attending elevation of insulin would be expected to result in increased expression and activity of ADD1/SREBP1. This is likely to affect both lipid accumulation in fat cells through increased expression of FAS, LPL, and other lipogenic genes, and more differentiation of adipocytes from precursor cells. Fig. 7 also illustrates that a possible effect on leptin expression could assert feedback regulation to the control of food intake. However, since there are no experiments yet illustrating that ADD1/SREBP1 can regulate endogenous leptin gene, this must be considered speculative.

Several interesting questions concerning the regulation of ADD1/SREBP1 by insulin remain. The robust regulation of this gene by feeding status and insulin suggests that other factors upstream of ADD1/SREBP1, as yet undefined, must be influenced by nutrients and hormones at the transcriptional and/or posttranscriptional levels. Second, there appears to be a mechanism for posttranscriptional regulation of ADD1/SREBP1 activity by insulin (Fig. 6, and reference 37). We have found recently that ADD1/SREBP1 is phosphorylated on serine residue(s) in response to insulin (our unpublished data). Whether this phosphorylation plays a role in the alteration of ADD1 activity remains to be determined.

A final question relates to the proteolytic processing of ADD1/SREBP1. This factor can be activated proteolytically on cultured fibroblasts in response to cholesterol depletion (15, 40, and references therein). For the endogenous protein to work effectively, it seems likely that there must be some means of removing it from the endoplasmic reticulum of fat tissue, as has been shown for the liver. However, since ADD1/SREBP1 expression is induced by feeding and reduced dramatically in fasting, paradoxical to what might be expected for a factor activated proteolytically by cholesterol depletion, it seems very likely that there are somewhat different mechanisms to activate this protein in fat. This could conceivably be through proteolysis linked to other signals, such as the levels of insulin or fatty acids, or this could conceivably involve escape from endoplasmic reticulum insertion through masking specifically the hydrophobic domain in the middle of ADD1/SREBP1. Further studies must address this question.

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