Upstream Stimulatory Factors Bind to Insulin Response Sequence of the Fatty Acid Synthase Promoter

USF1 IS REGULATED*

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Fatty acid synthase (FAS) plays a central role in de novo lipogenesis in mammals and birds. By the action of its seven active sites, FAS catalyzes all the reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS activity is exquisitely sensitive to nutritional, hormonal, and developmental status (1, 2). The concentration or activity of FAS in liver and adipose tissue changes dramatically when animals are subjected to nutritional and hormonal manipulations. We previously reported that due to changes in transcription, FAS synthesis declines and increases in an insulin-dependent manner during fasting and refeeding, respectively, and that insulin administration of streptozotocin-diabetic mice stimulates FAS transcription. We previously mapped the FAS insulin response sequence (IRS) to the proximal promoter region from position −71 to position −50, which contains an E-box DNA binding motif. Here, using competition gel shift assays and specific upstream stimulatory factor (USF) antibodies, we identified USF1 and USF2 as major components of complexes that bind to the FAS IRS. UV-cross-linking experiments further supported that USFs bind the FAS IRS. We also found that the amount of the 43-kDa USF1 was dramatically increased in liver of refed rats. In contrast, the amount of USF2 remained the same in liver of fasted or refeed rats. Moreover, a 17-kDa protein in both fasted and refeed rat liver was recognized by anti-USF1 antibodies, and this 17-kDa USF1-related protein was expressed in a manner opposite to that of the 43-kDa USF1, i.e. high in liver of fasted rats and decreased in liver of refeed rats. These data suggest that the regulation of USF expression may play an important role in the regulation of FAS transcription.

Fatty acid synthase (FAS) plays a central role in de novo lipogenesis in mammals and birds. By the action of its seven active sites, FAS catalyzes all the reaction steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate. FAS activity is exquisitely sensitive to nutritional, hormonal, and developmental status (1, 2). The concentration or activity of FAS in liver and adipose tissue changes dramatically when animals are subjected to different nutritional and hormonal manipulations. The rate of FAS synthesis declines when rats are fasted for 1–2 days while refeeding a high carbohydrate, fat-free diet increases synthesis of FAS (3). Increased circulating insulin and decreased glucagon levels may participate in regulation of FAS synthesis. We previously reported that FAS mRNA was not detectable in liver of fasted mice and that refeeding with a high carbohydrate diet dramatically increased the level of FAS mRNA, due to changes in the rate of FAS gene transcription (4, 5). The stimulation of FAS gene transcription by fasting/feeding was not observed in liver of streptozotocin-diabetic mice, in which FAS expression is detected at a very low level (5). Administration of insulin to streptozotocin-diabetic mice stimulated the level of FAS mRNA and FAS transcription rate (5). We also reported that insulin increased levels of FAS mRNA in 3T3-L1 adipocytes (4). Sequences mediating insulin induction of the FAS gene have been located to the first 332 base pairs of the FAS promoter by transient transfection of H4IIE hepatoma cells and 3T3-L1 adipocytes (6). We further mapped insulin response sequence (IRS) to the proximal promoter region from position −71 to position −50 by chimeric constructions of serial 5′-deletions of the rat FAS gene promoter ligated to the luciferase reporter gene and transfection into 3T3-L1 adipocytes (7). This IRS confers a stimulation of the FAS promoter activity by insulin, at physiological concentrations, in a dose-dependent manner. Moreover, we also demonstrated that three tandem repeats of IRS linked to a heterologous SV40 promoter were responsive to insulin. Both liver and adipocyte nuclear proteins bind to the FAS promoter, resulting in the IRS region being protected on DNase I footprinting analysis and specific band shift on a gel mobility shift assay (7). Neither the insulin response sequence from the amylase promoter (8, 9) nor that from the GAPDH promoter (10) displaced the binding of FAS IRS to the liver nuclear factor(s), suggesting that unique protein(s) are involved in the binding of FAS IRS and in the regulation of FAS gene transcription by insulin (7). Since these studies, we have been attempting to identify and isolate the transcription factors that bind to FAS IRS.

The upstream stimulatory factor (USF), belonging to the basic-helix-loop-helix (bHLH) family of transcription factors, was first identified for its involvement in transcription from the adenovirus major late (AdML) promoter (11–13). Purification of USF from HeLa cells has revealed that at least two different polypeptides, i.e. the 43-kDa USF1 and the 44-kDa USF2, contribute to USF activity in human cells (14). In addition to the human USF1 (15) and partial USF2 (16, 17) and murine USF2 (18), USF cDNA clones have been isolated from Xenopus (19) and sea urchin (20). Like other bHLH transcription factors such as Myc, E12, E47, and MyoD, USF binds to the "E-box" DNA binding motif, consisting of a canonical CANNNTG sequence. In addition to the bHLH motif, a leucine zipper (LZ) is immediately adjacent to bHLH and is also important for USF dimerization and DNA binding (15). The transcription activa-
tion domain of USF has been located at the N terminus of the protein and consists of two subregions (21). USF1 and USF2 have been found to be ubiquitously expressed, at various levels, in mammalian cells and bind to an E-box as homo- and heterodimers (16, 18). USF binding sites have been found in a number of genes (22–38), and USFs have been shown to participate in the transcriptional regulation of several of them. Two examples are the rat S14 gene (34) and liver-type pyruvate kinase (L-PK) gene (38), which are important in energy metabolism. Hepatic S14 mRNA in the rat can be increased by feeding a high carbohydrate, fat-free diet to the fasted rats for 12 h. Nuclear extracts (RNE in later parts of this paper) were prepared after refeeding a high carbohydrate, fat-free diet to the fasted rats for 12 h. Nuclear extracts (RNE in later parts of this paper) were prepared from Sprague-Dawley rats (Charles River) as described previously (7). Briefly, 80 μg of nuclear extracts were separated on a 12% SDS-PAGE and wet-transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore) with a Bio-Rad TransBlot cell. The membrane was blocked with blocking solution containing 1% bovine serum albumin, 1% milk and 1 × NET buffer (144 mM NaCl, 5 mM EDTA, 0.25% (w/v) gelatin, 0.05% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.4), incubated with primary antibodies (α-USF1 at 0.2 μg/ml or α-USF2 at 1:5000 dilution) in blocking solution, and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution in blocking solution), and developed with Western blot chemiluminescence reagent (DuPont NEN).

UV-cross-linking—20 μl of gel shift binding reaction as described above was exposed to UV light in a Bio-Rad Gene Linker for 10 min at a distance of 10 cm from the UV source. Then SDS loading buffer was added to 1 × concentration and reaction mixture applied to 12% SDS-PAGE. The gel was fixed with 10% acetic acid, 10% methanol and then exposed to x-ray films with intensifying screens.

RESULTS

FAS Insulin Response Sequence Contains an E-box to Which USF1 and USF2 Bind—We showed previously that the FAS gene promoter segment from –71 to –50 is the FAS IRS (7). This segment contains the sequence 5'-CATGTG-3' from position –65 to position –60, the E-box DNA binding motif of the bHLH family of transcription factors. Since the ubiquitous USF was initially identified by its ability to bind to the E-box within the AdML promoter, we investigated whether USFs were the hepatic factors that bind to the FAS IRS using gel mobility shift assays. The FAS IRS oligonucleotide containing the FAS promoter sequence from position –76 to position –50 was 32P-labeled by a Klenow fragment filling-in and incubated with RNE prepared from fasted and then refeed rat. Two major protein-DNA complexes, referred to as Band 1 and Band 2 in Fig. 1, were detected by gel mobility shift assay. The migration pattern of these two bands was found to be very similar, if not identical, to those formed by using end-labeled S14 and L-PK gene carbohydrate response elements as probes (Fig. 1). Since USF- or USF-related proteins bind to the E-box sequence of S14 and L-PK gene carbohydrate response elements, we tested whether these sequences compete for the nuclear factors binding to FAS IRS. As shown in Fig. 1, both S14 and L-PK carbohydrate response element oligonucleotides, as well as FAS IRS itself, could effectively compete for the formation of Band 1 and 2 at 20- and 40-fold molar excess to the labeled FAS IRS probe. Addition of the same amount of poly(d-c) to the gel mobility shift reactions had no such effect, suggesting that the competition detected under these conditions is specific. Oligonucleotides derived from the AdML promoter and mutation of the GCC upstream of the FAS IRS E-box to TAA could also effectively compete for the protein-DNA complexes in Band 1 and 2, while mutation of the E-box sequence CATGTG to ACTGGT and the sterol response element, the binding site for sterol response element-1-binding protein, did not (data not shown). These results suggest that similar USFs bind to the E-box of FAS IRS as well as S14 and L-PK gene carbohydrate response elements.

To further demonstrate that USFs bind to the FAS IRS, we tested whether Band 1 and 2 in Fig. 1 are affected by addition of USF-specific antibodies to the gel mobility shift assay. As shown in Fig. 2A, incubating RNEs with FAS IRS probe in the presence of anti-USF1 antibodies directed against the C-terminal 20 amino acids (position 291–310) completely disrupted.
these two major bands and resulted in “supershifts.” At the same time, a new protein-DNA complex (Band 3) appeared upon the addition of anti-USF1 antibodies. This newly appeared band did not appear to be caused by protein contaminants of the antibody preparation because gel shift assays with fixed amounts of anti-USF1 antibody and increasing amounts of nuclear extract (2–8 μg) resulted in a proportionally increased amount of Band 3 (Fig. 2B). This result demonstrates that 1) USF1 is a major component of the protein complexes that bind to FAS IRS and 2) within the nuclear extract there is at least one additional protein component that binds to the FAS IRS, probably by forming heterocomplexes with USF1.

Within the USF transcription factor family, USF2 is known to be able to heterodimerize with USF1 and has the same binding affinity to E-box sequences as USF1 (48). Currently, no other proteins have been reported to heterodimerize with USF1 and regulate gene transcription. To test whether USF2 is the other protein involved in binding to FAS IRS, we performed the following two experiments. First, to test the E-box specificity of Band 3, a gel mobility shift competition assay was carried out in the presence of anti-USF1 antibody and with S14 and L-PK oligonucleotides as competitors (Fig. 3A). Both S14 and L-PK carbohydrate response elements, at molar excesses of 20- and 40-fold, could compete for the formation of Band 3, as well as for the two supershifted bands. Compared to S14 sequence, L-PK sequence was found to compete less effectively. As expected, FAS IRS itself competed and nonspecific poly(dI-dC) did not. This result suggests that Band 3 represents an E-box-specific protein-DNA complex, which supports the probability that it contains USF2. The second experiment used rabbit polyclonal anti-USF2 antiserum in the gel shift assay to directly detect the presence of USF2 in Band 3. As shown in Fig. 3B, addition of anti-USF2, together with anti-USF1 antibody to the gel shift assay resulted in the total disappearance of Band 3, as well as the lower supershifted band (lane 4) formed by the addition of anti-USF1 antibody alone, demonstrating the presence...
ence of USF2 in Band 3. On the other hand, adding anti-USF2 antibody alone (lane 3, Fig. 3B) to the gel shift assay disrupted protein-DNA complex formation in both Band 1 and 2, suggesting USF2 is present in both of them. Taken together, we concluded that USF2 is the other major protein component that interacts and forms protein heterocomplexes with USF1 to bind FAS IRS.

To provide further supporting data for the involvement of USF1 and USF2 as major protein factors in the FAS IRS binding, we performed an UV-cross-linking experiment. The protein-DNA complexes formed under the same condition as in the gel shift assays with RNE were exposed to UV light, and the protein components cross-linked to the 32P-labeled FAS IRS probe were separated on SDS-PAGE and revealed by autoradiography. As shown in Fig. 4, major proteins cross-linked to the FAS IRS probe migrated at the 49-kDa position, and the free probe itself migrated at about 6 kDa. Subtraction of the probe size (6 kDa) from the cross-linked protein-DNA complex (49 kDa) gave the size of the protein to be 43 kDa, which is consistent with the size of USF1 (43 kDa) and USF2 (44 kDa in mouse and human but probably 42 kDa in rat; see descriptions below). As expected, addition of either FAS IRS itself or AdML promoter, but not poly(dI-dC), in 50- and 100-fold molar excesses to the UV-cross-linking reaction abolished the formation of the 49-kDa signal, demonstrating the specificity of the experiment.

Different Forms of USF1, but Not USF2, in Liver Nuclear Extracts from Fasted and Refed Rats Form Distinct FAS IRS Binding Complexes—We previously showed that in fasting animals transcription of FAS mRNA decreased and that in animals refed a high carbohydrate, fat-free diet the transcription of FAS mRNA dramatically increased. The stimulation of FAS transcription by refeeding is insulin-dependent and insulin administration to streptozotocin-diabetic animals increases FAS transcription (4, 5). Increased circulatory insulin level contributes to the increase of FAS transcription during refeeding. A simple way of regulating FAS transcription by this nutritional manipulation can be achieved by the modification of transcription factors, such as USF1 and USF2, which bind to the FAS IRS. To investigate the possible differences of USF1 and USF2 expression between fasted and refed rat liver, FNEs were prepared. Then gel shift assays of FNEs using FAS IRS as a probe were compared to same assays of RNEs. As shown in Fig. 5, FNEs exhibit a dramatically different pattern of the protein-DNA complexes. In contrast to the existence of Band 1...
and 2 formed by RNEs, Band 1 is missing and a faster migrating Band 4 appears. Addition of anti-USF1 and anti-USF2 antibodies to the FNE gel shift reactions resulted in "supershifts" and more importantly, retention of Band 3, which was undetermined. Since the anti-USF2 antiserum is highly specific for the detection of USF2,2 we strongly believe that the 42-kDa protein is the rat USF2. In contrast to the 43-kDa USF1, rat USF2 exists in equal amounts in RNE and FNE. The fact that the same amount of USF2 was detected in both nuclear extracts argues against the disappearance of 43-kDa USF1 and the greater amount of 17-kDa USF1-related protein in FNE is caused by general protein degradation during preparation of the nuclear extract. Overall, our data highly suggest that USF1, but not USF2, is regulated during fasting and refeeding, including the amount changes of the 43-kDa USF1 and the 17-kDa USF1-related protein.

To investigate whether the 17-kDa USF1-related protein binds to DNA, we carried out a UV-cross-linking experiment with FNEs (Fig. 7). In addition to the 49-kDa signal, as was found with the RNEs, an additional 23-kDa signal was also detected. Both the 49-kDa and 23-kDa signal could be specifically blocked by adding either cold FAS IRS or AdML promoter to the UV-cross-linking reaction, while addition of nonspecific poly(dl-dc) had no effect, demonstrating the specificity of the cross-linking experiment. Subtraction of the probe size (about 6 kDa) from the probe-linked protein size of the small band (23 kDa) gave the molecular mass of the protein to be about 17 kDa, which is consistent with the molecular mass of the small USF1-related protein.

**DISCUSSION**

Previously, we reported that the first 322 base pairs of the FAS promoter contained the insulin response element by transient transfection of H4IIE hepatoma cells and 3T3-L1 adipocytes.2 Subsequently, we mapped FAS IRS to the proximal promoter region from position −71 to position −50 by making chimeric constructions of serial 5′-deletions of the rat FAS promoter ligated to the luciferase reporter gene and transfection into 3T3-L1 adipocytes. This IRS sequence confers

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2 M. Sawadogo, personal communication.
about 3-fold increase of the reporter gene activity upon insulin treatment at physiological concentrations. Triple copies of FAS IRS fused to a heterologous SV40 promoter also exhibit insulin response. Since this identification of the cis-acting DNA element mediating insulin response of FAS, we have been making our efforts to identify and isolate the trans-acting factors. In this study, we have demonstrated that USF1 and USF2, which belong to the family of bHLH-LZ transcription factors, are major components of the protein complexes that bind to the E-box contained in FAS IRS. It was previously shown that USF1 can be functionally regulated in a redox-dependent manner (46). Sulfhydryl groups of the two cysteine residues, both present within the HLH protein–protein interface domain of USF1 and conserved among all known USF sequences from various species including human (15, 16), mouse (18), Xenopus (19), and sea urchin (20), are targets of this regulation in the DNA binding capacity, which can be translated into the modulation of USF1's ability to activate transcription (46). USF1 and USF2 are known to be expressed ubiquitously among various cells and tissues (18), based mainly on Northern blot analysis. Distributions of USF1 and USF2 among various tissues and their regulation under different physiological conditions have not been shown previously. In this report, we have demonstrated that USF1 protein in liver of fasted rats is very low and is markedly increased in liver of rats refeed a high carbohydrate, fat-free diet. In contrast, USF2 remains at the same concentration in liver of fasted or refeed rats. This observation suggests that these ubiquitous transcription factors are regulated at the protein level in response to physiological changes to control downstream gene expression.

Structure-function studies of the human 43-kDa USF1 revealed that the HLH and LZ regions are both important for USF oligomerization and DNA binding (15). Subsequently, transcription activation domain was localized to two regions at the N terminus (21). The first region is between positions 16 and 59 with a core between 26 and 39, and the second is between positions 93 and 156 with a core between 103 and 130.
Glucose and insulin can regulate a number of glycolytic and lipogenic enzymes. Their effects are dependent on each other in most cases. USFs have been reported to be involved in the glucose stimulation of L-PK and S14 transcription, and the involvement of USF1 and USF2 and their regulation have not been clearly addressed. Here we provide evidence for its binding to the FAS IRS. During fasting/freeing treatment of animals, both glucose and insulin control glycolytic and lipogenic enzyme expression. Their signal transduction pathways are different, but may converge at some points. Using common transcription factors such as USF may be one such intersection. The specificity of which genes are regulated by glucose and insulin may be contributed by the context of the DNA response element and/or protein interaction of USF with other transcription factors. In the case of rat S14 gene, although mutated glucose response element binds to the same protein complex containing USF in the gel shift assay in a manner indistinguishable from the wild-type glucose response element, it confers no glucose response in vivo (38). This observation suggests that other protein components are required to cooperate with USF and determine the glucose response specificity. In respect to the glucose response element sequence 5'-CACGTGNNNGCC-3'; it contains not only the E-box but also the downstream GCC, in which the CC dinucleotide in the E-box context are essential for a specific glucose response (38). FAS IRS contains the E-box sequence but not the downstream GCC even read from both strands, and the surrounding sequences may specify the insulin response. Further investigation will be necessary to elaborate the molecular mechanisms underlying the regulation of the glycolytic and lipogenic enzymes.

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