Sterol regulatory element-binding proteins (SREBPs) are transcription factors that are predominately involved in the regulation of lipogenic and cholesterogenic enzyme gene expression. To identify unknown proteins that interact with SREBP, we screened nuclear extract proteins with \(^{35}\)S-labeled SREBP-1 bait in Far Western blotting analysis. Using this approach, high mobility group protein-B1 (HMGB1), a chromosomal protein, was identified as a novel SREBP interacting protein.

In vitro glutathione S-transferase pull-down and in vivo coimmunoprecipitation studies confirmed an interaction between HMGB1 and both SREBP-1 and -2. The protein-protein interaction was mediated through the helix-loop-helix domain of SREBP-1, residues 309–344, and the A box of HMGB1. Furthermore, an electrophoretic mobility shift assay demonstrated that HMGB1 enhances SREBPs binding to their cognate DNA sequences. Moreover, luciferase reporter analyses, including RNA interference technique showed that HMGB1 potentiates the transcriptional activities of SREBP in cultured cells. These findings raise the intriguing possibility that HMGB1 is potentially involved in the regulation of lipogenic and cholesterogenic gene transcription.

Sterol regulatory element-binding proteins (SREBPs)\(^1\) are members of the basic helix-loop-helix leucine zipper (B-HLH-ZIP) family of transcription factors that bind to specific sterol-responsive elements (SREs) in the promoters of target genes and thereby regulate fatty acid and cholesterol synthesis (reviewed in Refs. 1–3). Two homologous genes, Srebp-1 and Srebp-2, have been identified. The Srebp-1 gene is further transcribed into two isoforms, SREBP-1c and -1a, by distinct promoters. In lipogenic organs such as liver and adipose tissues, the predominant isoform is SREBP-1c, which controls gene expression of lipogenic enzymes, including fatty acid synthase and ATP citrate lyase, whereas SREBP-2 plays a crucial role in the regulation of key enzymes in the cholesterol synthetic pathway such as 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase and farnesyl diphosphate synthase (4–9). However, the molecular mechanisms that determine this selectivity have not been entirely elucidated in the context of their preference for DNA sequence (10). It is likely related to their complicated interactions with various transcription factors and cofactors (11).

Several nuclear proteins that act as modulators of SREBP-regulated transcription have been identified. Some of them function as activators \((\text{e.g. CREB-binding protein (12), Sp1 (13), and NF-Y (14)) and some as repressors (e.g. YY1 (15, 16), ATF6 (17), and WT1 (18))}). Recently, we and others reported that hepatocyte nuclear factor 4 is another interacting factor (19, 20). Taken together, it has been demonstrated that SREBPs, like other transcription factors, interact with a broad range of proteins to control various signaling pathways. To better understand the precise mechanisms of SREBP regulation, a comprehensive study to identify unknown interacting factors is imperative.

In the present study, we set out to identify these proteins by screening nuclear extract proteins using Far Western blotting technique and identified high mobility group protein-B1 (HMGB1) as a new factor interacting with SREBP by matrix-assisted laser-desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry. HMGB1 is a chromatin-binding protein that belongs to high mobility group (HMG)-box family and appears to act as an architectural facilitator in the assembly of nucleoprotein complexes in a variety of DNA-related processes including transcription, replication, V(D)J recombination, and repair (reviewed in Refs. 21 and 22). Here, we describe the further function of HMGB1 in interacting with SREBPs.

**MATERIALS AND METHODS**

**Nuclear Protein Extraction**

Nuclear extracts from mice livers were prepared as previously described (23). Briefly, excised livers (0.5 g) from 24-h fasted or 12-h refed C57BL/6J mice were homogenized in a Polytron in 5 ml of buffer A (10 mM Hepes at pH 7.9, 25 mM KCl, 1 mM EDTA, 2 mM sucrose, 10% glycerol, 0.15 mM spermine, and 2 mM spermidine, supplemented with protease inhibitors (12.5 μg/ml N-acetyl-Leu-Leu-norleucinal (Calbiochem), 2.5 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml aprotinin). Pooled homogenate was then subjected to one stroke of a Teflon pestle in a Potter-Elvehjem homogenizer, followed by filtration through two layers of cheesecloth, and layered
over 10 ml of buffer A in a Beckman SW28 rotor. After centrifugation at 24,000 rpm for 1 h at 4 °C, the resulting nuclear pellet was resuspended in a buffer containing 10 mM Hepes at pH 7.9, 100 mM KC1, 2 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol supplemented with protease inhibitors, after which 0.1 volume of 5 mM NaCl was added. Each mixture was agitated gently for 30 min at 4 °C and then centrifuged at 89,000 rpm in a Himac S120AT2 rotor (Hitachi, Tokyo, Japan) for 30 min at 4 °C. The supernatant was used as nuclear extract.

**Communoprecipitation**

Aliquots of nuclear extract protein (100 μg) were diluted in binding buffer (20 mM Hepes at pH 7.9, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with protease inhibitors) and were then boiled briefly in SDS-PAGE sample buffer to elute proteins for subsequent electrophoresis.

**Western Blotting**

Following SDS-PAGE, immunoprecipitated proteins were transferred onto nitrocellulose membrane (Hybond ECL, Amersham Biosciences). SREBP-1 and HMGB1 were detected using a 1:1000 dilution of rabbit anti-SREBP-1 or rabbit anti-HMGB1 (BD Pharmingen) antibodies, respectively, in TBS buffer (20 mM Tris-HCl at pH 7.6 and 140 mM NaCl) containing 0.2% Tween 20 (Bio-Rad) and 5% skimmed milk. Bound antibodies were detected with an horseradish peroxidase-coupled anti-rabbit IgG secondary antibody (Amersham Biosciences) and visualized using ECL chemiluminescent substrates (Amersham Biosciences). For Western blot experiments, immunoprecipitation was performed using mouse monoclonal antibody to avoid background signals from immunoprecipitation antibody detected by secondary antibody.

**Far Western Blotting**

Nuclear extract or immunoprecipitated protein was blotted onto polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences), was incubated for 1 h at room temperature in blocking buffer (TBS buffer containing 0.2% Tween 20 and 5% skimmed milk) and was then mixed overnight at 4 °C with [35S]Methionine-labeled SREBP-1c bait. [35S]Methionine-labeled SREBP-1c bait was generated by using an in vitro transcription/translation system using reticulocyte lysate (TNT T7 quick-coupled transcription/translation system, Promega). The visualized band from immunoprecipitation antibody detected by Far Western Blotting was excised, destained, washed, and then counted using a liquid scintillation counter.

**Post-source Decay Analysis**

To confirm the protein identification, PSD spectra were acquired at delayed extraction and reflectron mode. The accelerating voltage was set at 20 kV, grid voltage at 75%, guide wire voltage at 0.024%, and delay time at 100 ns. The timed ion selector was pre-set to the [M+H] mass of the peptides. The spectra were acquired in 10–13 segments with mirror ratios 1.0–0.13 and assembled by using Doorstep software (DataExplorer). Obtained peptide masses were used to search the NCBI data base by using the MS-Tag algorithm (prospector.ucsf.edu).

**Plasmid Constructions**

A series of truncated derivatives of mouse SREBP-1c cDNA expression plasmids used for in vitro transcription/translation in GST pull-down assays were constructed by inserting each fragment into pcDNA3.1 (+) expression vector (Invitrogen) as follows: SREBP-1-FL (amino acid residues 1–453) cDNA fragment was cut out of pTREX-SREBP-1c construct described previously (26) by NotI and XhoI followed by insertion into pcDNA3.1 (+) at NotI and XhiI sites; SREBP-1-B-HLH-ZIP (residues 1–364) and SREBP-1-B-HLH (residues 1–344) were constructed by amplifying each segment by PCR with a 5′ primer, including NheI site and initiation codon (ATG), and 3′ primers, including a stop codon (TAA) and SalI site, and by inserting it into NheI and SalI sites, respectively (26). The SREBP-1-A-HLH-ZIP (residues 1–285) was constructed by inserting NotI-AgeI fragment attached with an adapter, including Age I site and a stop codon into pcDNA3.1 (+) at NotI and XbiI (also blunted) sites; SREBP-1-A-HLH-ZIP (residues 1–256) was constructed by ligating NotI-AvrII (AvrII site was blunted with Klenow DNA polymerase) fragment into pcDNA3.1 (+) at NotI and XbiI and XhoI sites of pcDNA3.1 (+). SREBP-1-A-ΔTA (residues 91–460) was described previously (20). Transmission from these expression plasmids was driven by T7 promoter when used in TnT T7 quick-coupled transcription/translation system (Promega).

A prokaryotic expression plasmid with His6 tag at N-terminal used for generating SREBP-1c recombinant protein was constructed as follows: full-length SREBP-1c cDNA fragment cut out of pTREX-SREBP-1c construct described previously (26) by NotI and XhoI and blunted at both ends with Klenow DNA polymerase was inserted into NotI (blunted) and SmaI sites of pIEX2.4d (Roche Applied Science), where NotI sites were blunt-ended in order for the frame not to be shifted. To construct an expression plasmid for GST-SREBP-1 fusion protein, low:full-length SREBP-1-B-HLH-ZIP (amino acid residues 1–285) cDNA fragment (residues 1–285) was constructed by inserting Stul-EcoRI (blunted) fragment into NcoI site (blunted) of pIEX2.4d. These plasmids were used for generating recombinant proteins both in BL21(DE3) Escherichia coli and in vitro transcription/translation system (Promega). To construct an expression plasmid for GST-HMBG-1 fusion protein, EcoRI fragment was inserted into pGEX-4T2 (Amersham Biosciences) at the EcoRI site. All the constructs were verified by sequencing.

**Generation of Recombinant Proteins**

[35S]Methionine-labeled proteins were generated from pcDNA3.1 (+) expression plasmids (Invitrogen) using T7 RNA polymerase and reticulocyte lysate (TnT T7 quick-coupled transcription/translation system, Promega) or from pIEX2.4d plasmids utilizing E. coli lysate with T7 RNA polymerase (Rapid Translation System, Roche Applied Science) according to the manufacturer’s protocol. GST and GST fusion proteins were expressed in E. coli (DH5α) using pGEX-4T (Amersham Biosciences) and purified using glutathione-Sepharose beads (Amersham Biosciences) and was then calibrated internally within 20 ppm using instrument software (DataExplorer).
Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed as previously described (27). Briefly, the DNA probe was prepared by annealing two oligonucleotides, 5'-CACACACCTCGGGGAGGAGTTTGCAGTGGGGTG-3' and 5'-CACACACCTGCAAACTCCTCCC-3', which correspond to both strands of the sterol regulatory element (SRE) in the promoter of low density lipoprotein receptor gene (28), and labeling them with [α-32P]dCTP by filling in the 5'-overhangs with Klenow DNA polymerase (Amersham Biosciences), followed by purification on Sephadex G-50 (Amersham Biosciences) columns. The labeled DNA probe was incubated with recombinant His-SREBP-1 or -2 protein (200 ng) mixed with various amount of GST-HMG1 or GST alone in a buffer containing 10 mM Hepes at pH 7.9, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, supplemented with protease inhibitors). After 2 h of incubation at 4 °C, the beads were washed four times in wash buffer (20 mM Hepes at pH 7.9, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 supplemented with protease inhibitors), and were boiled briefly in SDS-PAGE sample buffer to elute proteins for subsequent electrophoresis. Gels were dried, and signals were visualized with BAS2000 imaging system (Fuji photo film).

Preparation of Short Hairpin RNA Expression Cassette for RNAi Analysis

The iGENE algorithm (iGENE Therapeutics) was used to list candidates of RNA interference target sequences for human HMG1. For six candidates short hairpin RNA expression plasmids were constructed by cloning annealed oligonucleotides into pENTR/U6 expression vector (BLOCK-iT U6 RNAi Entry Vector Kit, Invitrogen). The most effective construct was selected through transfection experiments (data not shown). The selected target sequence was 5'-AGACCTGAGAATG-3', which was cloned into pENTR/U6 vector, and the final construct was generated by cloning annealed oligonucleotides into pENTR/U6 expression vector.

RESULTS

Far Western Blot Demonstrating That SREBP-1 Binds to Several Nuclear Proteins in the Liver—In an attempt to search for new interacting factors with SREBP, we first performed Far Western blotting analysis of nuclear proteins extracted from mouse livers. As shown in Fig. 1a, several bands were detected with 35S-labeled SREBP-1c (nuclear form) bait. To further examine whether these proteins are communoprecipitated with SREBP-1 using anti-SREBP-1 antibody, immunoprecipitates with a mouse monoclonal antibody were subjected to immunoblotting with a rabbit polyclonal antibody. c, Far Western analysis of SREBP-1-coimmunoprecipitated proteins. The same proteins as in b were blotted and incubated with [35S]methionine-labeled SREBP-1c. SREBP-1c was detected by its homodimerization.
HMGB1 was identified with by far the highest probability. The other two bands were also identified as other proteins. To reinforce this identification, post-source decay (PSD) analyses of amino acid sequence were also performed. Three parent ions denoted by #1 through #3 in Fig. 2b were further analyzed in PSD mode, and spectra were assembled by the instrument software (Fig. 2c). By searching the NCBI database with the MS-Tag algorithm (prospector.ucsf.edu), the three precursor ions were all identified as HMGB1.

In Vivo Coimmunoprecipitation of HMGB1 by Anti-SREBP-1 Antibody—To validate this identification of the band observed by Far Western as HMGB1, the in vivo coimmunoprecipitated nuclear proteins by anti-SREBP-1 antibody were immunoblotted using anti-HMGB1 antibody (Fig. 3). To avoid background signals from secondary antibody detecting immunoprecipitation antibody, immunoprecipitation was performed with mouse monoclonal antibody and immunoblotting was with rabbit polyclonal antibody. Because fasted mouse livers contain barely detectable levels of SREBP-1, they were used as a negative control. As expected, the HMGB1 band was detected only in refed mice where SREBP-1 existed abundantly and thereby coimmunoprecipitated HMGB1, whereas fasted mice liver exhibited no HMGB1 bands. These results demonstrate that SREBP-1 binds to HMGB1 in vivo in the nuclei of hepatocytes.

SREBP-1 Interacts with HMGB1 through HLH Domain—We further investigated the binding of SREBP-1 to HMGB1 by GST pull-down assay. As expected, the full-length nuclear form of SREBP-1c (amino acid residues 1–453) generated and labeled with [35S]methionine by in vitro transcription/translation system were pulled down by recombinant GST-HMGB1 fusion protein (Fig. 4c). In contrast, GST control resin showed no interaction. Next we attempted to determine the domain of SREBP-1 responsible for the interaction with HMGB1; SREBP-1 consists of several domains such as acidic, basic (B), helix-loop-helix (HLH), and leucine zipper (ZIP) domains (Fig. 4a). For this purpose, deletion derivatives of SREBP-1c were generated and labeled with [35S]methionine using the in vitro transcription/translation system and were subjected to GST pull-down assay with GST-HMGB1 (Fig. 4, d and e). In the series of deletion derivatives sequentially deleted from the C-terminal, SREBP-1c with amino acid residues 1–344 or longer (with HLH domain (309–344)) were pulled down by GST-HMGB1, whereas those with amino acid residues 1–309 or shorter (without HLH domain) were not. These findings demonstrate that HLH domain of SREBP-1 is critical for its interaction with HMGB1.

SREBP-1 and -2 are homologous with each other, and in particular, their HLH domains have the highest similarity of 77% in their amino acid sequence (Fig. 4f). Therefore, we examined whether SREBP-2 also binds to HMGB1 in the same pull-down assay. As expected, SREBP-2 was pulled down with GST-HMGB1 (Fig. 4g), demonstrating that SREBP-2 as well as SREBP-1 binds to HMGB1.

HMGB1 Interacts with SREBP-1 through its A Box—To precisely understand the interaction between SREBP-1 and HMGB1 and at the same time to map the site of HMGB1 required for this interaction, we performed another GST pull-down assay in the reverse setting using a GST-SREBP-1 fusion protein containing B-HLH-ZIP domain of SREBP-1 (Fig. 5b), and recombinant proteins with deleted domains of HMGB1. HMGB1 is structured into two HMG box domains (A and B boxes, 30% identical in amino acid sequence) plus a highly
FIG. 2—continued

PSD Spectrum

1510.7066
Parent mass

Fragment Mass

400 - 1100

900 - 1400

MSSY
AF
FF
VQ
TCR

RCTQVFF
A
Y
S
SM

Theoretical fragments

| b ion series | 104.05 | 219.28 | 306.36 | 469.54 | 540.62 | 687.8 | 834.98 | 934.11 | 1062.24 | 1163.35 | 1137.57 | - |
| sequence | M | S | S | Y | A | F | F | V | Q | T | C | R |
| y ion series | - | 1380.58 | 1293.5 | 1206.42 | 1043.24 | 972.16 | 824.98 | 677.81 | 578.67 | 450.54 | 349.43 | 175.21 |
| y11 | y10 | y9 | y8 | y7 | y6 | y5 | y4 | y3 | y2 | y1 |

Peptide fragments of HMGB1 identified by PSD

#1 1128.5944: YEXDIAAYR163
#2 1510.6950: MSSYAFFVQTCR24
#3 2109.9957: GEMWNNATAADDKQPYEK146

Fig. 2—continued
acidic C-terminal region (Fig. 5a). We therefore generated the following three 35S-labeled recombinant proteins with in vitro transcription/translation system: full-length HMGB1 protein, HMGB1-Abox containing A box alone, and HMGB1-Abox lacking A box and containing B box and C-terminal. To avoid carryover of HMGB1 protein included in the reaction reagent from reticulocytes, we used an E. coli lysate system. As expected, HMGB1 exhibited specific binding to B-HLH-ZIP domain of SREBP-1 (Fig. 5c). It was further revealed that the A box of HMGB1 was sufficient for this binding, whereas protein without A box (ΔAbox) was not capable of binding (Fig. 5c). These data demonstrate that the A box of HMGB1 and the B-HLH-ZIP domain of SREBP-1 have a direct interaction.

**HMGB1 Increases SREBP DNA Binding**—As a transcription factor, SREBP-1 binds to specific sequences, i.e. SRE (5'-ATCCACCCAC-3'). To examine the effect of HMGB1 on the DNA binding ability of SREBP-1, electrophoretic mobility shift assay (EMSA) was conducted using recombinant His-SREBP-1c and 32P-labeled DNA probe with SRE sequence. As shown in Fig. 6b, a single band with retarded migration by SREBP-1c was detected (lane 2). The specificity of this binding was confirmed by its supershift with anti-SREBP-1 antibody (lane 3) and by lack of binding of GST-HMGB1 alone to the probe (lane 4). When GST-HMGB1 fusion protein was added, the intensities of bands were enhanced in a dose dependent manner (lanes 5–7), but not by GST alone (lane 8), indicating that HMGB1 stimulated the formation of SREBP-1-SRE complex. It was also remarkable that GST-HMGB1 did not change the mobility, suggesting that HMGB1 was not included in the SREBP-1-SRE complex. The same results as above were obtained with SREBP-2 (Fig. 7). These data demonstrate that HMGB1 promotes the binding of SREBP to SRE sequence.

To examine which HMGB1 domain contributes to this activation, we performed EMSA using deletion derivatives of HMGB1 (Fig. 8a). Addition of recombinant full-length His-HMGB1 protein enhanced the formation of SREBP-1-SRE complex in a dose-dependent manner, whereas addition of His-HMGB1-Abox and -ΔAbox did not (Fig. 8b). This suggests that A and B boxes are both required to promote the formation of the SREBP-SRE complex.

**FIG. 3.** *In vivo* coimmunoprecipitation of HMGB1 with SREBP-1. Coimmunoprecipitation of HMGB1 with SREBP-1 using anti-SREBP-1 antibody was shown. Anti-SREBP-1-immunoprecipitates from hepatic nuclear proteins (100 μg) was resolved by SDS-PAGE followed by Western blotting analysis with an anti-HMGB1 antibody. Nuclear extract proteins were prepared from livers of mice in a fasted (F) and refed (R) state.

**FIG. 4.** GST pull-down assays to determine the HMGB1 binding site of SREBP-1. a, schemes of functional domains of nuclear SREBP-1c and deletion derivatives were shown. b, schematic representation of a GST-HMGB1 fusion protein used in our pull-down assays. c–e, 35S-labeled recombinant SREBP-1c proteins of various length generated by in vitro transcription/translation system were pulled down by GST-HMGB1 fusion protein. Proteins pulled down as well as 4% of input were electrophoresed and visualized by BAS2000. Truncated proteins devoid of helix-loop-helix domain were shown not to be pulled down. f, schematic representation of homology between SREBP-1 and -2. Helix-loop-helix domain shows the highest similarity of 77% among their domains. g, Full-length nuclear forms of both SREBP-1 and -2 were demonstrated to be pulled down by GST-HMGB1.
Luciferase Reporter Analyses Demonstrating That HMGB1 Activates SREBP in Vivo—To estimate the impact of HMGB1-SREBP interaction on the transcriptional activity of SREBP in the cells, series of transfection experiments were performed using a plasmid containing luciferase reporter gene driven by a SREBP-responsive promoter (pSRE-Luc) and HMGB1 expression and/or RNA interference (RNAi) plasmids. As shown in Fig. 8 (a and b), when HMGB1 expression was increased by co-transfection of its expression plasmid, the transcriptional activity of SREBP-1c or -2 as assessed through pSRE-Luc was significantly enhanced. Conversely, when endogenous HMGB1 expression was knocked down by RNAi, the SREBP activity was reduced in parallel. Moreover, when the effect of RNAi was counteracted by HMGB1 expression plasmid that is resistant against the RNAi because it does not contain the RNAi target sequence we selected in the 3’ non-coding region, the SREBP activity was partially restored. These functional studies demonstrate that HMGB1 potentiates the transcriptional activities of SREBP in cells, indicating that HMGB1-SREBP interaction is physiologically relevant in vivo.

HMGB1 Promotes SREBP Dimerization—As described above, we demonstrated that HMGB1 interacts with SREBPs through their HLH domains. Because HLH domains are known to play a crucial role in the homodimerization of SREBP-1 (30–32), we examined the effect of HMGB1 on the dimerization of SREBP-1 using the TR-FRET assay system. FRET from donor to acceptor fluorescence molecule can be observed when the two come in close proximity of each other. We first tested the binding of His-tagged full-length HMGB1, HMGB1-Abox, or HMGB1ΔH9004-Abox to GST-SREBP-1 with this system; HMGB1 proteins were labeled with anti-His antibody conjugated with donor fluorescence (europium), whereas GST-SREBP-1 and GST alone were labeled with anti-GST antibody conjugated with acceptor fluorescence (allophycocyanin), and thereafter both were mixed and TR-FRET from donor to acceptor was measured on a fluorometer. Reiterating the conclusion from the GST pull-down assay, FRET was observed in the case of full-length HMGB1, HMGB1-Abox, or HMGB1ΔH9004-Abox to GST-SREBP-1 with this system; HMGB1 proteins were labeled with anti-His antibody conjugated with donor fluorescence (europium), whereas GST-SREBP-1 and GST alone were labeled with anti-GST antibody conjugated with acceptor fluorescence (allophycocyanin), and thereafter both were mixed and TR-FRET from donor to acceptor was measured on a fluorometer. We next examined whether dimerization of SREBP-1 could be detected by FRET between His-SREBP-1 and GST-SREBP-1, which were labeled with donor (europium) and acceptor (allophycocyanin) fluorescence-conjugated antibody, respectively. As shown in Fig. 9b, addition of HMGB1 increased FRET between His-SREBP-1 and GST-SREBP-1, suggesting that HMGB1 promotes the dimerization of SREBP-1.

FIG. 5. GST pull-down assays to map the SREBP-1 binding site of HMGB1. a, schematic representation of domain structure of HMGB1 and full-length or truncated versions of His-tagged HMGB1 proteins. b, schema of GST-SREBP-1 fusion protein used in pull-down assays. c, series of 35S-labeled recombinant HMGB1 deletion derivatives were pulled down by GST alone or GST-SREBP-1 fusion protein. FL, full-length HMGB1; Abox, truncated HMGB1 protein containing A-box alone; ΔAbox, deleted HMGB1 protein lacking A-box.

FIG. 6. EMSAs demonstrating HMGB1 enhancement of SREBP binding to SRE oligonucleotides. a, Coomassie Brilliant Blue (CBB) staining of recombinant His-tagged SREBP-1 and -2 proteins used in our EMSA. These proteins were expressed in E. coli and purified with nickel-bound resin. b and c, EMSA with SRE probe detecting recombinant SREBP-1 (b) and -2 (c) (each 200 ng). The 32P-labeled oligonucleotides were incubated with the indicated recombinant proteins and/or antibody and resolved on a 4.6% polyacrylamide gel. Lanes 2 and 5–7 show increasing amounts of GST-HMGB1 protein enhances DNA binding of SREBP in a dose-dependent manner.
DISCUSSION

Our present studies clearly demonstrate that SREBPs interact with HMGB1, which enhances their binding to cognate DNA sequences and thereby transcriptional activity. HMGB1 is a very abundant chromatin-binding protein, acting as an architectural facilitator in a variety of DNA-related processes, including transcription, replication, V(D)J recombination, and repair (reviewed in Refs. 21 and 22). In fact, HMGB1 has been...
implicated in the regulation, via both activation and repression, of transcription. The mechanisms are considered to be by interacting both with the basal transcription machinery (33) and with individual transcription factors such as Hox proteins (34), p53 (35), NF-κB (36), and steroid hormone receptors (37). In the latter case, HMGB1 enhances their binding to the cognate DNA sites. Our current findings presented SREBP as another example of HMGB1 interaction with transcription factors.

In our series of experiments, we found out that the HLH domain of SREBP-1 interacts with HMGB1. Because this domain is well known to be committed to the dimerization of SREBP-1 (30–32), we examined the effects of HMGB1 on the process, and obtained the intriguing result that HMGB1 promotes the dimerization of SREBP-1. It is tempting to speculate that this effect might contribute to the enhanced binding of SREBP to SRE, whereas it is generally considered that DNA bending induced by HMGB1 facilitates the DNA-protein interaction (22, 38). These mechanisms are not exclusive and might perhaps be cooperative.

Recently a new paradigm has been proposed that nuclear architectural proteins like HMGB1 are involved in the fast switching of gene expression (38). Supporting this, mutant yeast lacking HMGB1-related proteins, the non-histone proteins NHP-6A and -6B, are severely retarded in the activation of several inducible genes (39, 40). In this context, it is very intriguing that SREBPs, especially SREBP-1, play an important role in the induction of genes encoding lipogenic enzymes, which are the most vigorously up-regulated in liver and adipose tissue when animals are refed after starvation (26, 29). Our present findings thus raise the possibility that HMGB1 might be potentially involved in this dynamic regulation of lipogenic genes.

It has been documented that homozygous knock-out mice of the Hmgb1 gene are born alive, but die within 24 h due to hypoglycemia (41). When glucose is given parenterally, Hmgb1-deficient mice survive for several days. These mice are reported to be completely lacking in adipose tissue. This phenotype of lipodystrophy might be related to its interaction with SREBP-1, because SREBP-1 and lamin A/C deficiency, where the interaction of SREBP-1 and lamin A/C is reported (42). Although further studies are needed, our present finding that SREBPs interact with HMGB1 might provide good clues for exploring the molecular mechanisms regulating lipogenesis and adiposity.

Our data demonstrated that SREBP-1 binds to HMGB1 through its HLH domain, which leads us to speculate that many other HLH-type transcription factors could interact with HMGB1. In fact, we have already found that TFE3, the member closest to SREBP in the B-HLH-ZIP family, binds to HMGB1 by Far Western analysis (data not shown), and other members might also interact with HMGB1. Investigation of these interactions between HMGB1 and HLH proteins will give...
valuable insights into yet undiscovered roles of HMGB1 in the regulation of transcription.

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REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1997) Cell 89, 331–340
2. Brown, M. S., and Goldstein, J. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11041–11048
3. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) Cell 100, 391–398
4. Hua, X., Yokoyama, C., Wu, J., Briggs, M., Brown, M., Goldstein, J., and Wang, X. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11603–11607
5. Bennett, M. K., Lopez, J. M., Sanchez, H. B., and Osborne, T. F. (1995) J. Biol. Chem. 270, 25578–25583
6. Ericsson, J., Jackson, S. M., Lee, B. C., and Edwards, P. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 945–950
7. Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S., and Goldstein, J. L. (1997) J. Clin. Invest. 99, 846–854
8. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., and Shimano, H. (1998) J. Clin. Invest. 101, 2331–2339
9. Sato, R., Okamoto, A., Inoue, J., Miyamoto, W., Sakai, Y., Emoto, N., Shimano, H., and Maeda, M. (2000) J. Biol. Chem. 275, 12497–12502
10. Amemiya-Kudo, M., Shimano, H., Hasty, A. H., Yahagi, N., Yoshihikawa, T., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Sato, R., Kiumura, S., Ishibashi, S., and Yamada, N. (2002) J. Lipid Res. 43, 1220–1235
11. Magana, M. M., Kuo, S. H., Towe, H. C., and Osborne, T. F. (2000) J. Biol. Chem. 275, 4726–4733
12. Olner, J. D., Andreassen, J. M., Hansen, S. K., Zhou, S., and Tjian, R. (1996) Genes Dev. 10, 2903–2911
13. Sanchez, H. B., Yieh, L., and Osborne, T. F. (1995) J. Biol. Chem. 270, 1161–1169
14. Ericsson, J., Jackson, S. M., and Edwards, P. A. (1996) J. Biol. Chem. 271, 24559–24564
15. Bennett, M. K., Ngo, T. T., Athanakar, J. N., Rosenfeld, J. M., and Osborne, T. F. (1999) J. Biol. Chem. 274, 13025–13032
16. Ericsson, J., Usheva, A., and Edwards, P. A. (1999) J. Biol. Chem. 274, 14508–14513
17. Zeng, L., Lu, M., Mori, K., Luo, S., Lee, A. S., Zhu, Y., and Shyy, J. Y. (2004) EMBO J. 23, 2960–2965
18. Rae, F. K., Martinez, G., Gillinder, K. R., Smith, A., Shooter, G., Forrest, A. R., Grimmmond, S. M., and Little, M. H. (2004) Oncogene 23, 3067–3079
19. Misawa, K., Horita, T., Arimura, N., Hirano, Y., Inoue, J., Emoto, N., Shimano, H., Shimizu, M., and Sato, R. (2003) J. Biol. Chem. 278, 21203–21212
20. Yamamoto, T., Shimano, H., Nakagawa, Y., Ide, T., Yahagi, N., Matsuzaka, T., Nakakuki, M., Takahashi, A., Suzuki, H., Sone, H., Toyoshima, H., Sato, R., and Yamada, N. (2004) J. Biol. Chem. 279, 12027–12035
21. Bustin, M. (2002) Sci. STKE 2002, E39
22. Thomas, J. O., and Travers, A. A. (2001) Trends Biochem. Sci. 26, 167–174
23. Sheng, Z., Otani, H., Brown, M. S., and Goldstein, J. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 935–938
24. Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S., and Goldstein, J. L. (1996) J. Clin. Invest. 98, 1575–1584
25. Shimano, H., Shimomura, I., Hammer, R. E., Herz, J., Goldstein, J. L., Brown, M. S., and Horton, J. D. (1997) J. Clin. Invest. 100, 2115–2124
26. Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shinonori, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999) J. Biol. Chem. 274, 35832–35839
27. Amemiya-Kudo, M., Shimano, H., Yoshihikawa, T., Yahagi, N., Hasty, A. H., Okazaki, H., Tamura, Y., Shinonori, F., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Sato, R., Kiumura, S., Ishibashi, S., and Yamada, N. (2000) J. Biol. Chem. 275, 31078–31085
28. Briggs, M., Yokoyama, C., Wang, X., Brown, M., and Goldstein, J. (1993) J. Biol. Chem. 268, 14490–14496
29. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5987–5992
30. Ferre-D’Amare, A. R., Prendergast, G. C., Ziff, E. B., and Burley, S. K. (1993) Nature 363, 38–45
31. Parraza, A., Bellesleli, L., Ferre-D’Amare, A. R., and Burley, S. K. (1998) Structure 6, 661–672
32. Rishi, V., Gal, J., Krylov, D., Fridriksson, J., Boysen, M. S., Mandrup, S., and Vinas, C. (2004) J. Biol. Chem. 279, 11863–11874
33. Ge, H., and Roeder, R. G. (1994) J. Biol. Chem. 269, 17136–17140
34. Zappavigna, V., Falciola, L., Helmer-Citterich, M., Mavilio, F., and Bianchi, M. E. (1996) EMBO J. 15, 4981–4991
35. Jayaraman, L., Moorthy, N. C., Murthy, K. G., Manley, J. L., Bustin, M., and Prives, C. (1998) Genes Dev. 12, 462–472
36. Brickman, J. M., Adam, M., and Ptashne, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10679–10683
37. Bonnyaratanakorakit, V., Melvin, V., Prendergast, P., Altmann, M., Ronfani, L., Bianchi, M. E., Tarasaviciene, L., Nordeen, S. K., Allegretto, E. A., and Edwards, D. P. (1998) Mol. Cell. Biol. 18, 4471–4487
38. Bianchi, M. E. (1999) EMBO Rep. 1, 109–114
39. Moreira, J. M., and Holmberg, S. (2000) J. Biol. Chem. 275, 25832–25839
40. Fragiadakis, G. S., Tzamarias, D., and Alexandraki, D. (2004) EMBO J. 19, 233–242
41. Calogero, A., Grassi, F., Aguzzi, A., Voigtländer, T., Ferrier, P., Ferrari, S., and Bianchi, M. E. (1999) Nat. Genet. 22, 276–280
42. Lloyd, D. J., Trembath, R. C., and Shackleton, S. (2002) Hum. Mol. Genet. 11, 769–777