THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 30, Issue of July 29, pp. 27523–27532, 2005

Printed in U.S.A.

High Mobility Group Protein-B1 Interacts with Sterol Regulatory Element-binding Proteins to Enhance Their DNA Binding*

Received for publication, December 27, 2004, and in revised form, April 21, 2005
Published, JBC Papers in Press, May 12, 2005, DOI 10.1074/jbc.M414549200§

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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 (*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 pH7.9, 25 mM KCl, 1 mM EDTA, 2 M 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

* This work was supported by a grant from the Research Fellowships of the Japan Society for the Promotion of Science (to N. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SREBP, sterol regulatory element-binding protein; B-HLH-ZIP, basic helix-loop-helix leucine zipper; SRE, sterol-responsive element; HMG, high mobility group; HMGB1, high mobility group protein-B1; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; TBS, Tris-buffered saline; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; PSD, post-source decay; TR-FRET, time-resolved fluorescence resonance energy transfer.

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 KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol supplemented with protease inhibitors, after which 0.1 volume of 5 M 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.

Coimmunoprecipitation

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, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 12.5 μg/ml *N*-acetyl-Leu-Leu-norleucinal, and 2 μg/ml leupeptin as final concentration), and were subjected to immunoprecipitation using a rabbit polyclonal or a mouse monoclonal anti-SREBP-1 antibody (24, 25) and protein G-Sepharose beads (Amersham Biosciences). After 6-h 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 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 polyclonal anti-SREBP-1 and 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 ^{35}S-labeled protein bait in the same buffer. [^{35}S]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), and was directly added to the buffer. After washing three times with TBS buffer containing 0.2% Triton X-100, signals were visualized with BAS2000 (Fuji photo film, Tokyo, Japan).

Protein Identification by MALDI-TOF MS

Peptide Mass Fingerprinting—Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The visualized band was excised, destained, washed four times in wash buffer (50% acetonitrile and 25 mM NH_4HCO_3 at pH 8.0), reduced in reducing buffer (100 mM dithiothreitol and 100 mM NH_4HCO_3) at 56 °C for 30 min, and alkylated with 100 mM acrylamide (in 100 mM NH_4HCO_3) at 37 °C for 30 min. The gel slice was then dehydrated with acetonitrile (high-performance liquid chromatography-grade, Wako Chemicals) and rehydrated with 10 μl of 50 mM NH_4HCO_3 (pH 8.0) containing 10 μg/μl modified trypsin (sequencing grade, Promega), and trypsin in-gel digestion was carried out for 8 h at 37 °C. The resulting peptides were extracted with 66% acetonitrile-0.1% trifluoroacetic acid (high-performance liquid chromatography-grade, Wako Chemicals), condensed by vacuum centrifugation, and desalted by ZipTipC18 pipette tips (Millipore). Following elution into 2 μl of 60% acetnitrile-0.1% trifluoroacetic acid, 0.5 μl was applied to a target plate, overlaid with 0.5 μl of α-cyano-4-hydroxycinnamic acid (Sigma) matrix, and evaporated at room temperature. Matrix-assisted laser-desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry was performed using a mass spectrometer (Voyager-DE STR, Applied Biosystems) operated in delayed extraction and reflectron mode. The obtained mass spectrum was calibrated externally using standard peptides (des-ARG-bradykinin, [Glu-1]fibrinopeptide B, and adrenocorticotropic hormone (clip 1–17): *m/z* = 904.4681, 1570.6774, and 2093.0867, respectively, PE biosystems) and was then calibrated internally within 20 ppm using trypsin autolytic peptides present in the sample (*m/z* = 842.5100, 1045.5642, and 2211.1046). Obtained data of monoisotopic ion mass ([M+H]) were entered into the National Center for Biotechnology Information (NCBI) data base, searching for corresponding protein by MS-Fit algorithm (prospector.ucsf.edu).

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 instrument software (DataExproler). 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 pTK-SREBP-1c construct described previously (26) by NotI and XbaI followed by insertion into pcDNA3.1(+) at NotI and XbaI 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 XhoI sites of pcDNA3.1(+); SREBP-1-Basic (residues 1–309) 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 XbaI sites; SREBP-1-Δ(B-HLH-ZIP) (residues 1–285) was constructed by ligating NotI-AvrII (AvrII site was blunted with Klenow DNA polymerase) fragment into pcDNA3.1(+) at NotI and XbaI (also blunted) sites; SREBP-1-C-terminal (residues 365–449) was PCR-amplified with 5′-primer, including an initiation codon and inserted into EcoRI and XhoI sites of pcDNA3.1(+). SREBP-1-ΔTA (residues 91–460) was as described previously (20). Transcription 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 His_6 tag at N-terminal used for generating SREBP-1c recombinant protein was constructed as follows: full-length SREBP-1c cDNA fragment cut out of pTK-SREBP-1c construct described previously (26) by NotI and XbaI and blunted at both ends with Klenow DNA polymerase was inserted into NotI (blunted) and SmaI sites of pIVEX2.4d (Roche Applied Science), where NotI sites were blunted in order for the frame not to be shifted. To construct an expression plasmid for GST-SREBP-1 fusion protein, cDNA corresponding to amino acid residues 286–364 (basic helix-loop-helix leucine zipper region) was PCR-amplified and inserted into pGEX-4T2 (Amersham Biosciences) at BamHI and SalI sites without frameshift. A full-length SREBP-2 cDNA construct in pcDNA3.1(+) was described previously (20).

To construct HMGB1 expression plasmids, mouse full-length HMGB1 cDNA reverse-transcribed from liver total RNA was amplified by PCR and cloned into pGEM-T-easy vector (Promega). After sequencing the plasmid, full-length cDNA fragment cut out by EcoRI and blunted at both ends was inserted into XhoI sites (blunted) of pIVEX2.4d to construct His-HMGB1-FL. For His-HMGB1-Abox (amino acid residues 1–97), EcoRI (cut and blunted), StuI fragment was cloned into XhoI site (blunted) of pIVEX2.4d. Similarly, His-HMGB1-ΔAbox (amino acid residues 98–215) was constructed by inserting StuI-EcoRI (blunted) fragment into NcoI site (blunted) of pIVEX2.4d. Those plasmids were used for generating recombinant proteins both in BL21(DE3) *Escherichia coli* and in *in vitro* transcription/translation system (Rapid Translation System, Roche Applied Science). To construct an expression plasmid for GST-HMGB1 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

[^{35}S]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 pIVEX2.4d 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) by standard method. His-tagged proteins were expressed with constructs using pIVEX2.4d in BL21(DE3) strain of *E. coli* and purified by binding to Ni-IDA resin (ProBond Purification System, Invitrogen) and eluting with imidazole followed by dialysis. Full-length HMGB1 protein without His tag was obtained with protease cleavage followed by treatment with benzamidine-Sepharose (Amersham Biosciences).

GST Pull-down Assays

GST pull-down assays were performed according to standard protocol. Briefly, GST or GST fusion proteins attached onto glutathione-Sepharose beads (Amersham Biosciences) in advance were mixed with an [^{35}S]methionine-labeled target proteins in binding buffer (20 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).

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′-GAGATCACCCCACTGCAAACTCCTCCC-3′ and 5′-GCAGGGGGAGGAGTTTGCAGTGGGGTG-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-^{32}P]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 a recombinant His-SREBP-1 or -2 protein (200 ng) mixed with various amount of GST-HMGB1 or GST alone in a buffer containing 10 mM Hepes at pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 30 μg/ml poly(dI-dC), and 0.1% Triton X-100, for 30 min on ice. The DNA·protein complexes were resolved on 4.6% polyacrylamide gels in 1× TBE buffer or glycine buffer (50 mM Tris-HCl at pH 8.0, 380 mM glycine, and 2 mM EDTA). Supershift reactions were performed by adding anti-SREBP-1 or -2 polyclonal antibody.

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 HMGB1. 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′-AGACCTGAGAATGTATCCCCAAA-3′ on the 3′ non-coding region and annealed oligonucleotides were 5′-CACCACTTGAGAGTGTATCTCCAACGTGTGCTGTCCGTTGGGGATACATTCTCAGGT-3′ and 5′-AAAAACCTGAGAATGTATCCCCAACGGACAGCACACGTTGGAGATACACTCTCAAGT-3′.

Transfection and Luciferase Assays

Luciferase reporter plasmid with sterol regulatory element (pSRE-Luc) was previously described (7). Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin. Cells were transfected with pSRE-Luc and the indicated expression plasmids using SuperFect Transfection Reagent (Qiagen). After a 24-h incubation, the luciferase activity in transfectants was measured on a luminometer by using a standard assay kit (Promega).

Measurement of Time-resolved Fluorescence Resonance Energy Transfer

For detecting interaction between SREBP-1 and HMGB1, a TR-FRET assay was performed by incubating 10 nM GST-SREBP-1 or GST alone, and 10 nM allophycocyanin-conjugated anti-GST antibody (SureLight, PerkinElmer Life Sciences) with 0–100 nM His-HMGB1-FL, His-HMGB1-Abox, or His-HMGB1-ΔAbox recombinant protein and 1 nM europium-conjugated anti-His_6 antibody (Lance, PerkinElmer Life Sciences) in FRET assay buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 0.1% bovine serum albumin, supplemented with protease inhibitors) for 8 h at 4 °C. Time-resolved fluorescence exited at 340 nm and detected at 665 nm with delay time of 50 μs was measured by cumulative counts of 1000 repeats on an ARVO MX analyzer (PerkinElmer Life Sciences). The results are expressed as 1000 × (665 nm/615 nm). For experiments examining SREBP-1 dimerization, similar TR-FRET assays between GST-SREBP-1 and His-SREBP-1 (10 nM each) with or without 1 nM no-tagged-HMGB1 were performed.

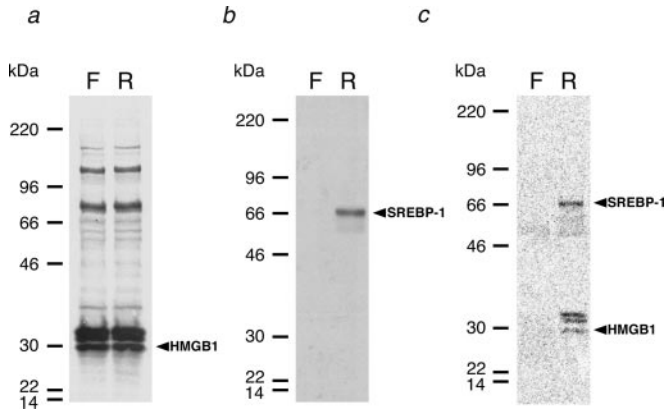


FIG. 1. **Far Western blotting analysis detecting hepatic nuclear proteins associated with SREBP-1c.** *a*, Far Western analysis of mice hepatic nuclear extracts using ^{35}S-labeled SREBP-1c as a bait. Aliquots of nuclear proteins (25 μg) extracted from fasted (*F*) and refed (*R*) mice livers were subjected to SDS-PAGE. [^{35}S]Methionine-labeled SREBP-1c bait was generated by *in vitro* transcription/translation system. *b*, immunoprecipitation of SREBP-1 from nuclear proteins (100 μg) with 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-coimmunoprecitated proteins. The same proteins as in *b* were blotted and incubated with [^{35}S]methionine-labeled SREBP-1c. SREBP-1 was detected by its homodimerization.

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. 1*a*, several bands were detected by ^{35}S-labeled SREBP-1c (nuclear form) bait. To further examine whether these proteins are coimmunoprecipitated with SREBP-1 using anti-SREBP-1 antibody, immunoprecipitated nuclear proteins were analyzed by Far Western technique in the same way. Because hepatic SREBP-1 is scarce when animals are fasted and is markedly induced when animals are refed after starvation as reported previously (29) and shown in Fig. 1*b*, we compared nuclear proteins from refed mouse livers with those from fasted animals as a negative control. As shown in Fig. 1*c*, ^{35}S-labeled SREBP-1c bait detected three bands around 30–35 kDa only in refed mice, suggesting that these nuclear proteins physiologically interact with SREBP-1c. Homodimerization of SREBP-1 was also detected at 66 kDa, which demonstrates that this Far Western blotting analysis is equally sensitive and specific with Western blotting.

Identification of the Protein as HMGB1 Using MALDI-TOF MS—Next we proceeded to identification of these nuclear proteins. The three strong bands were noticed by Ponceau-staining of crude nuclear bands exactly at the same positions as by Far Western analysis. Therefore, total nuclear proteins were resolved by SDS-PAGE, stained with Coomassie Brilliant Blue (Fig. 2*a*). The three corresponding bands were excised, applied to in-gel trypsin digestion and analyzed by peptide mass fingerprinting method using MALDI-TOF MS.

Fig. 2*b* shows a representative MALDI-TOF MS spectrum obtained from a trypsin-digested sample of the lowest band (around 30 kDa). These dozens of peaks were analyzed with MS-fit (prospector.ucsf.edu) peptide mass fingerprinting algorithm, and a protein named high mobility group protein-B1

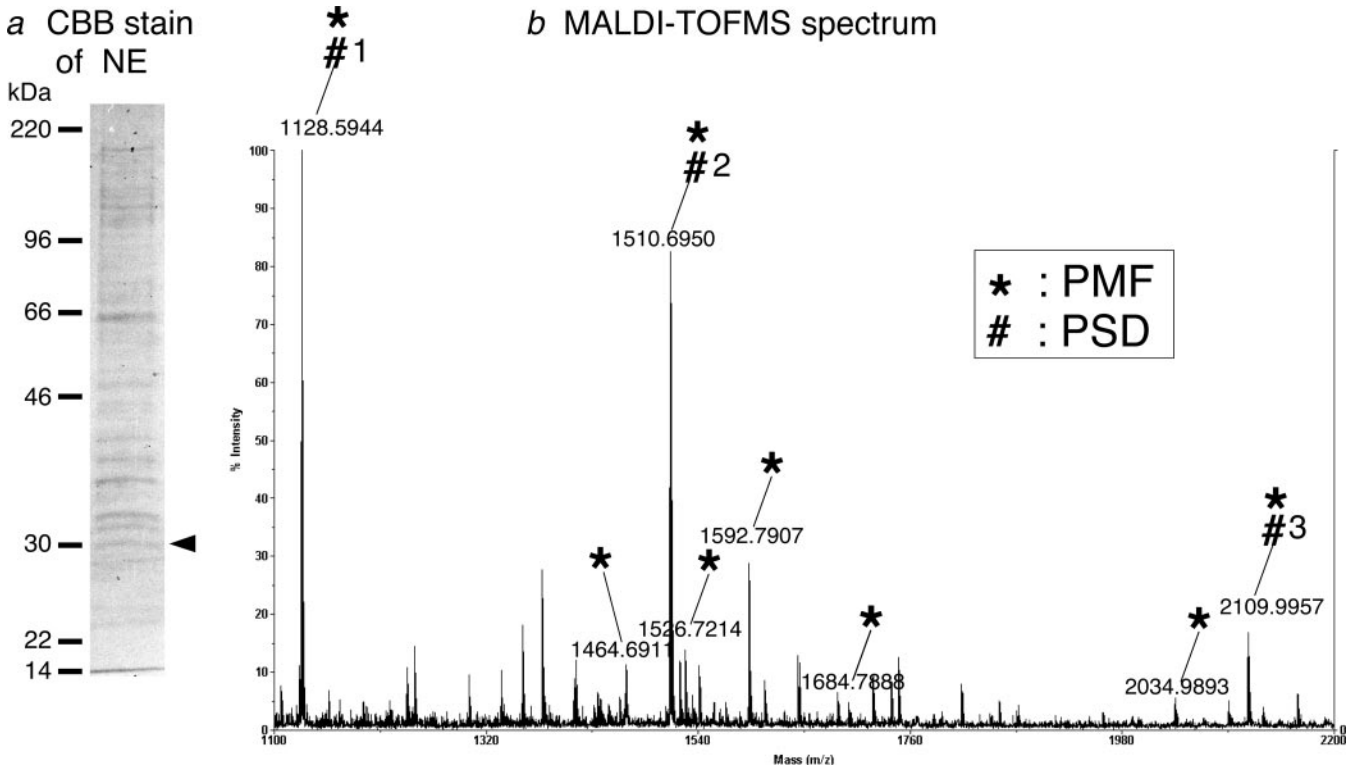


FIG. 2. **Protein identification by MALDI-TOF MS.** *a*, Coomassie Brilliant Blue (*CBB*) staining of hepatic nuclear proteins resolved by SDS-PAGE. The band indicated by the *arrowhead* was excised and subjected to trypsin digestion followed by peptide mass analyses. *b*, a representative spectrum of trypsin-digested peptides obtained on a MALDI-TOF MS analyzer. Peaks denoted by the *asterisk* were identified as originating from HMGB1 by standard peptide mass fingerprinting (*PMF*) algorithm (MS-Fit). *c*, spectra resulted from post-source decay (*PSD*) analysis. Three parent ions denoted by the # *symbol* in *b* were further analyzed in PSD mode, and a representative composite mass spectrum of fragment ions from a precursor of 1510.7 Da, denoted by *#2* in *b*, is displayed. Series of *b* and *y* ions (b4–b9 and y7–y10) were detected, and a partial amino acid sequence (MSSYAFFVQTCR, corresponding to HMGB1 amino acid residues 13–24) was determined. Similarly, precursors denoted by *#1* and *#3* were revealed to be HMGB1 amino acid residues 155–163 and 129–146, respectively.

(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. 2*b* were further analyzed in PSD mode, and spectra were assembled by the instrument software (Fig. 2*c*). By searching the NCBI data base 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.

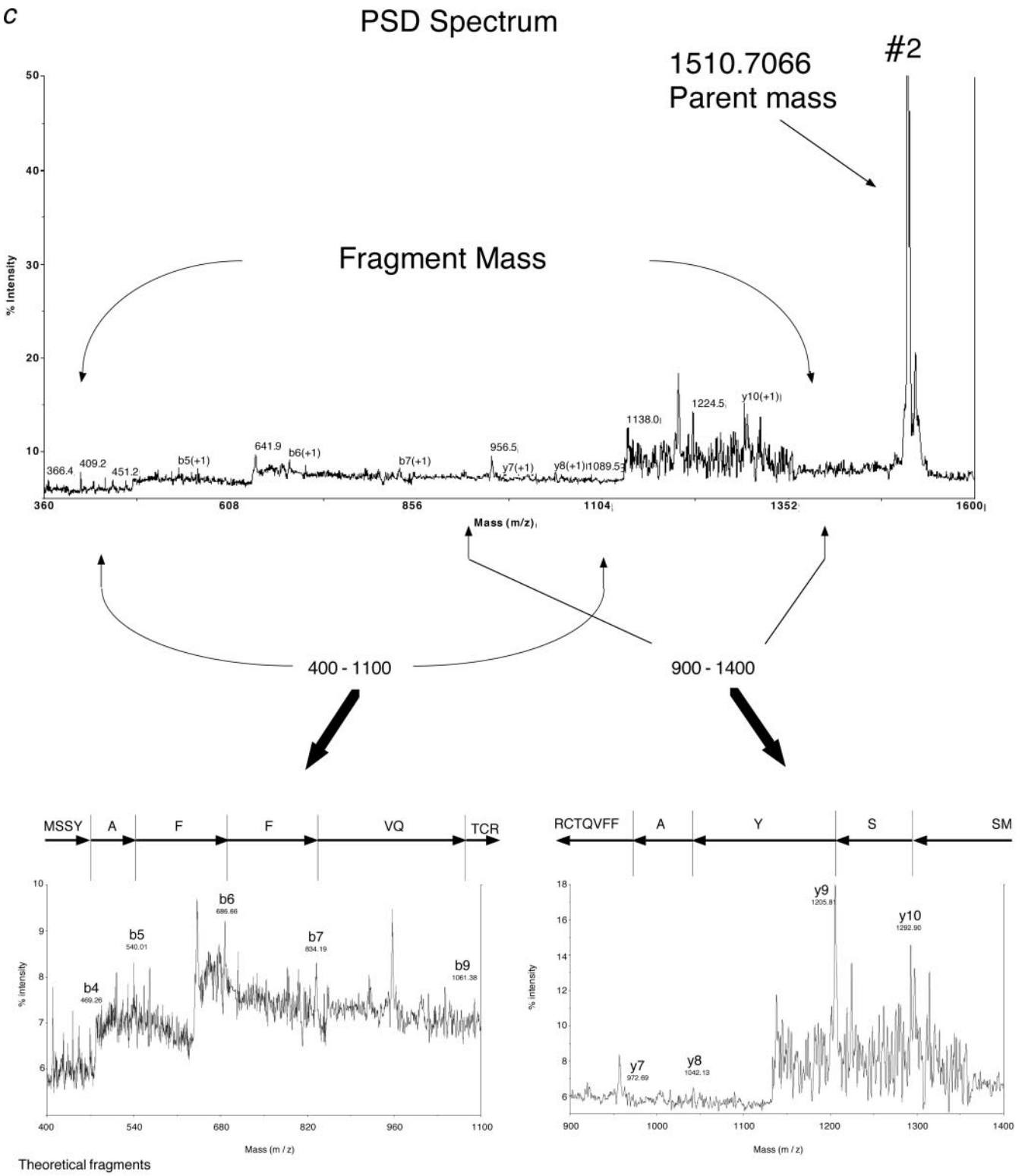
SREBP-1 Interacts with HMGB1 through HLH Domain—We further investigated the binding of SREBP-1 to HMGB1 by GST pull-down assay. As expected, the full-length nuclear form of SREBP-1c (amino acid residues 1–453) generated and labeled with [^{35}S]methionine by *in vitro* transcription/translation system were pulled down by recombinant GST-HMGB1 fusion protein (Fig. 4*c*). 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. 4*a*). For this purpose, deletion derivatives of SREBP-1c were generated and labeled with [^{35}S]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. 4*f*). 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. 4*g*), 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. 5*b*), 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

c



Theoretical fragments

	b1	b2	b3	b4	b5	b6	b7	b8	b9	b10	b11	
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

	m / z	sequence start		end
#1	1128.5944 :	155	YEKDIAAYR	163
#2	1510.6950 :	13	MSSYAFFVQTCR	24
#3	2109.9957 :	129	LGEMWNNTAADDKQPYEK	146

FIG. 2—*continued*

acidic C-terminal region (Fig. 5*a*). We therefore generated the following three ^{35}S-labeled recombinant proteins with *in vitro* transcription/translation system: full-length HMGB1 protein, HMGB1-Abox containing A box alone, and HMGB1-ΔAbox 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. 5*c*). 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. 5*c*). These data demonstrate that the A box of HMGB1 and the B-HLH-ZIP domain of SREBP-1 have a direct interaction.

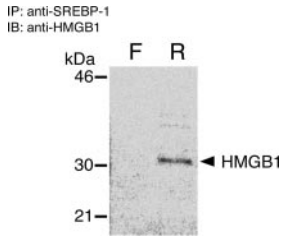


FIG. 3. ***In vivo* coimmunoprecipitation of HMGB1 with SREBP-1.** Coimmunoprecipitation of HMGB1 with SREBP-1 using anti-SREBP-1 antibody was shown. Anti-SREBP-1-immunoprecipitates from hepatic nuclear proteins (100 μg) was resolved by SDS-PAGE followed by Western blotting analysis with an anti-HMGB1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

HMGB1 Increases SREBP DNA Binding—As a transcription factor, SREBP-1 binds to specific sequences, *i.e.* SRE (5′-ATCACCCCAC-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 ^{32}P-labeled DNA probe with SRE sequence. As shown in Fig. 6*b*, 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. 8*a*). 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. 8*b*). This suggests that A and B boxes are both required to promote the formation of the SREBP·SRE complex.

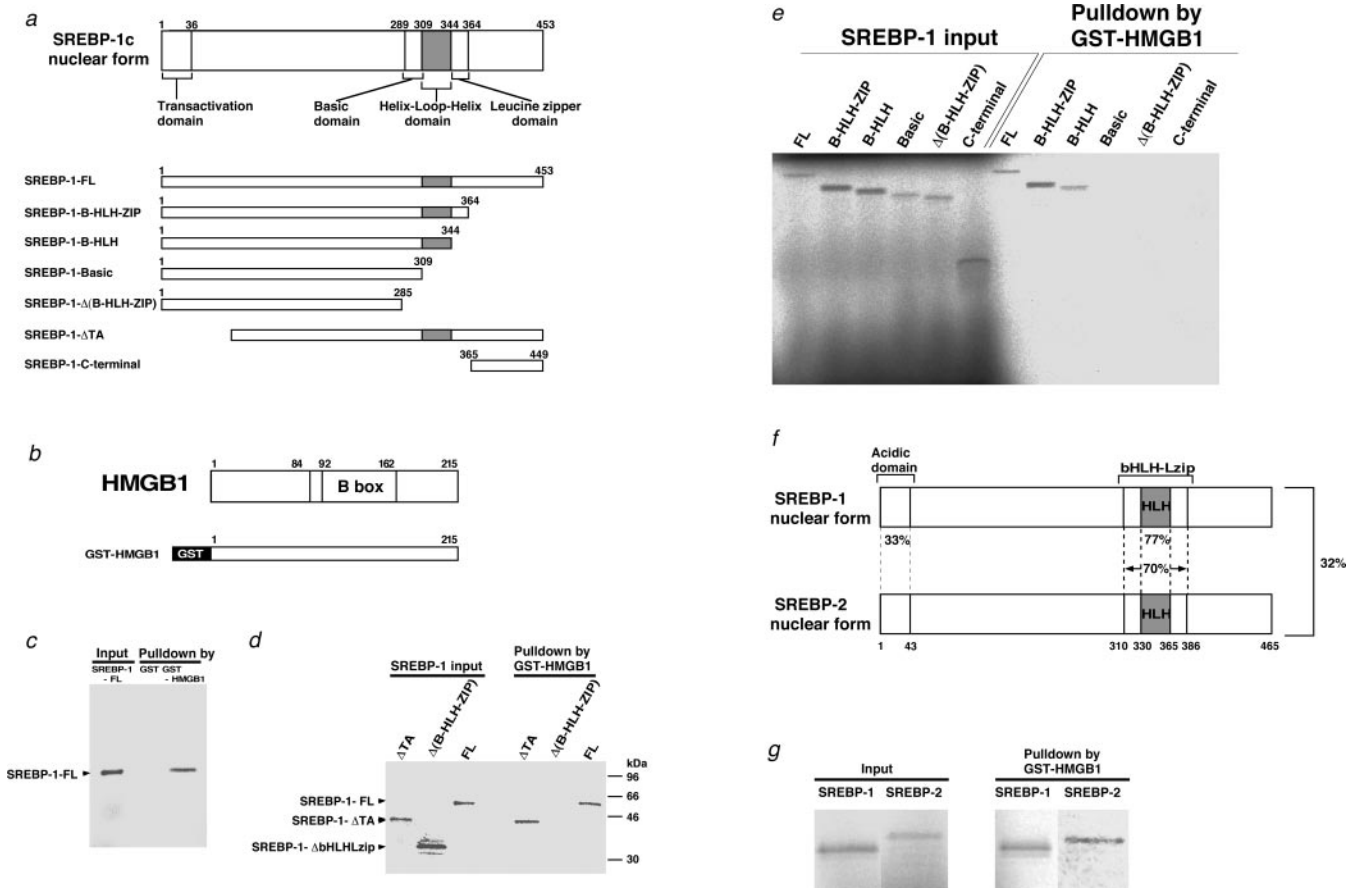


FIG. 4. **GST pull-down assays to determine the HMGB1 binding site of SREBP-1.** *a*, schemes of functional domains of nuclear SREBP-1c and deletion derivatives were shown. *b*, schematic representation of a GST-HMGB1 fusion protein used in our pull-down assays. *c–e*, ^{35}S-labeled recombinant SREBP-1c 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 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 countered 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*.

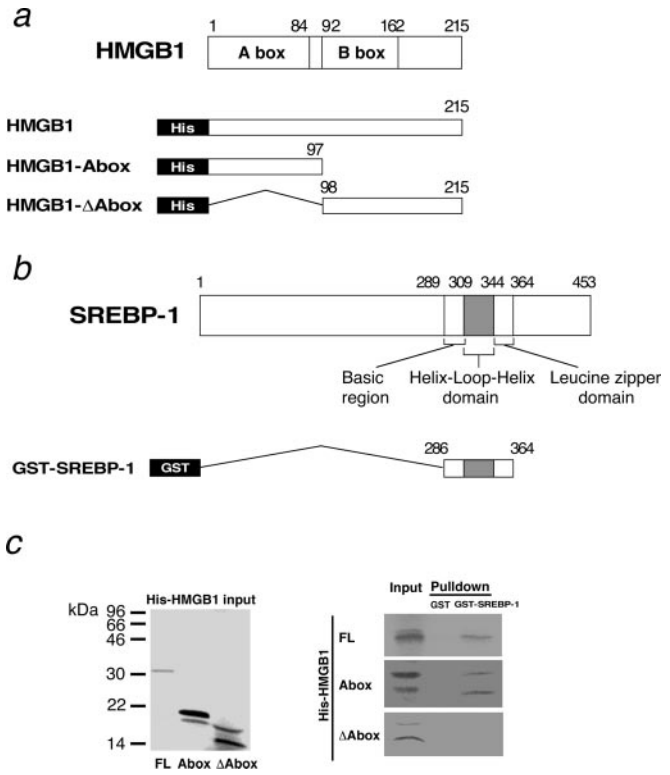


FIG. 5. **GST pull-down assays to map the SREBP-1 binding site of HMGB1.** *a*, schematic representation of domain structure of HMGB1 and full-length or truncated versions of His-tagged HMGB1 proteins. *b*, schema of GST-SREBP-1 fusion protein used in pull-down assays. *c*, series of ^{35}S-labeled recombinant 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; Δ*Abox*, deleted HMGB1 protein lacking A box.

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 HMGB1-ΔAbox 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 or HMGB1-Abox, whereas no FRET occurred with HMGB1-ΔAbox (Fig. 9*a*).

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. 9*b*, addition of HMGB1 increased FRET between His-SREBP-1 and GST-SREBP-1, suggesting that HMGB1 promotes the dimerization of SREBP-1.

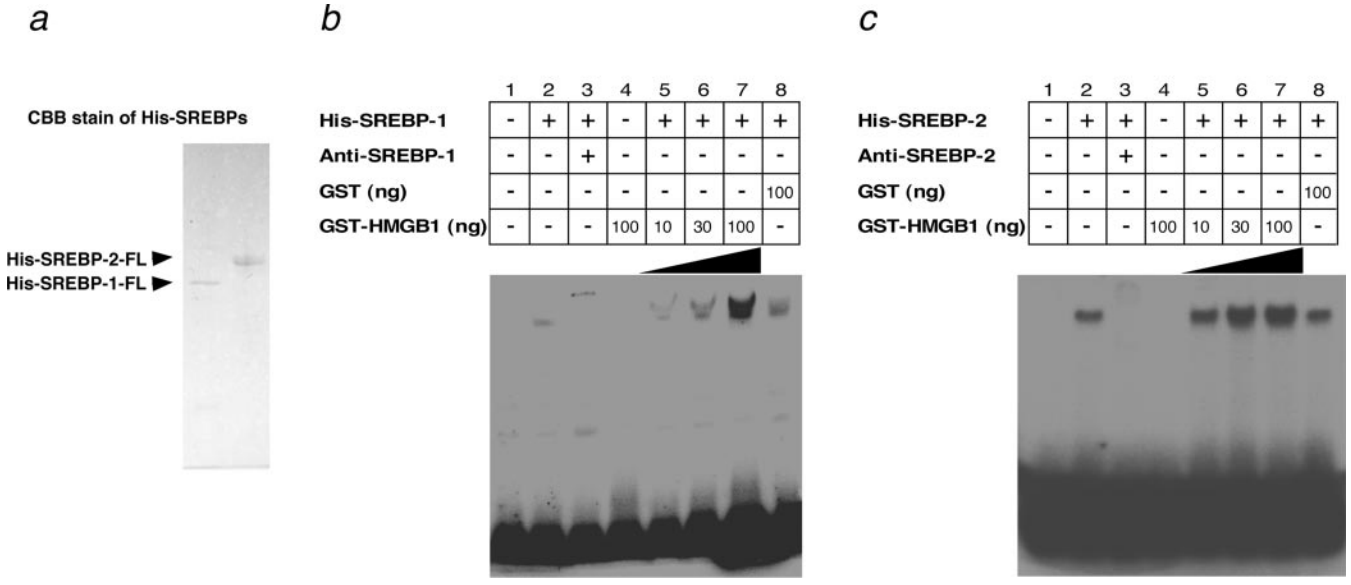


FIG. 6. **EMSAs demonstrating HMGB1 enhancement of SREBP binding to SRE oligonucleotides.** *a*, Coomassie Brilliant Blue (*CBB*) staining of recombinant His-tagged SREBP-1 and -2 proteins used in our EMSA. These proteins were expressed in *E. coli* and purified with nickel-bound resin. *b* and *c*, EMSA with SRE probe detecting recombinant SREBP-1 (*b*) and -2 (*c*) (each 200 ng). The ^{32}P-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.

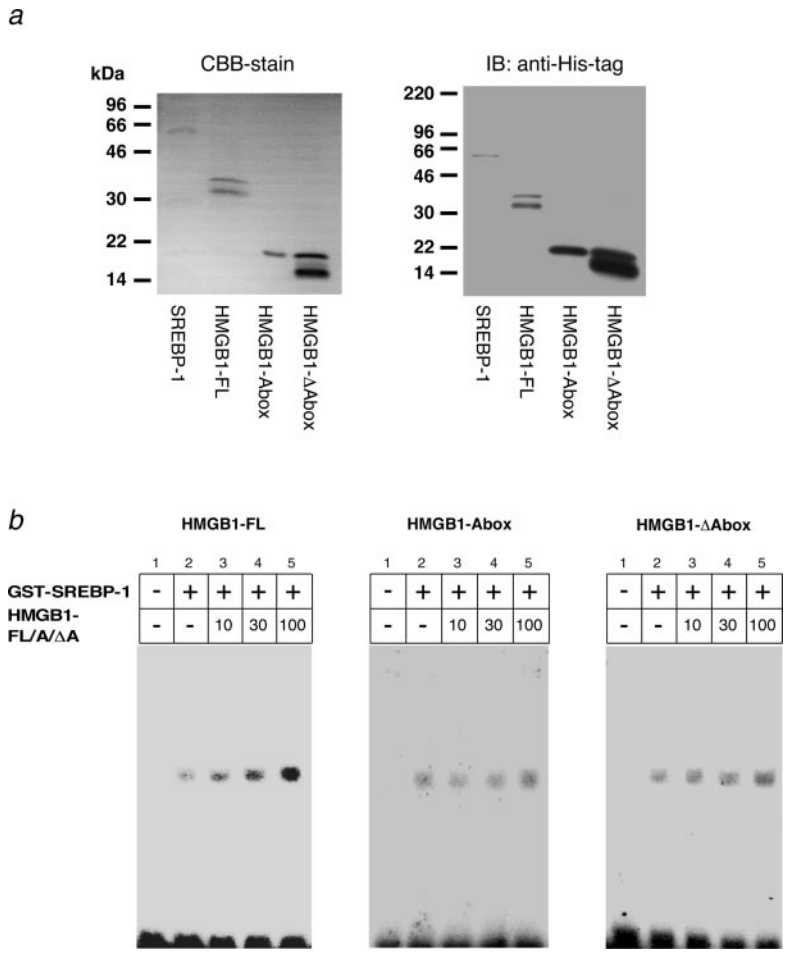


FIG. 7. **EMSAs showing that full-length HMGB1 is required to promote the formation of SREBP·SRE complex.** *a*, Coomassie Brilliant Blue (*CBB*) staining (*left panel*) and Western blotting with anti-His-tag antibody (*right panel*) of recombinant His-tagged HMGB1 proteins used in this experiment. *b*, EMSA with SRE probe and recombinant GST-SREBP-1 protein mixed with various length of HMGB1. The differential effects of full-length HMGB1 (*left panel*) *versus* HMGB1 containing A box alone (*middle panel*) and HMGB1 lacking A box (*right panel*) on DNA binding of GST-SREBP-1 are shown.

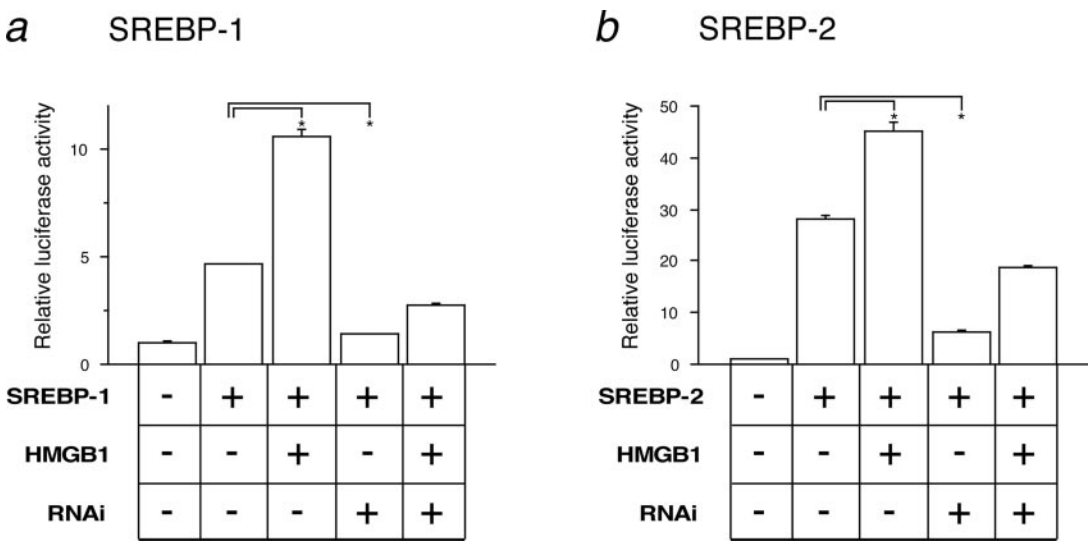


FIG. 8. **Luciferase reporter analyses demonstrating that HMGB1 activates SREBP *in vivo*.** *a* and *b*, the effects of increase or decrease in HMGB1 amount on SREBP-1c (*a*) or -2 (*b*) transcriptional activities as assessed by luciferase reporter analysis. Human embryonic kidney 293 cells were transfected with pSRE-Luc (0.25 μg) and pcDNA3.1-SREBP-1c or -2 expression plasmid (0.025 μg) together with the indicated HMGB1 expression/RNAi plasmids (1.0 μg each). As an expression plasmid for HMGB1, pcDNA3.1-HMGB1 was used, whereas the control contains the same amount of empty pcDNA3.1 plasmid. To decrease the endogenous HMGB1, short hairpin RNA expression plasmid (pENTR/U6-HMGB1), designed to evoke RNA interference against HMGB1 mRNA at a locus in the 3′ non-coding region (therefore not interfering with mRNA expressed by pcDNA3.1-HMGB1), was co-transfected (control is the same amount of pENTR/U6-lacZ, a short hairpin RNA expression plasmid targeting for lacZ). After a 24-h incubation, the luciferase activity was measured on a luminometer. *, significance at $p < 0.01$ by two-tailed Student's *t* test against control. *Bars*, ± S.E.

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

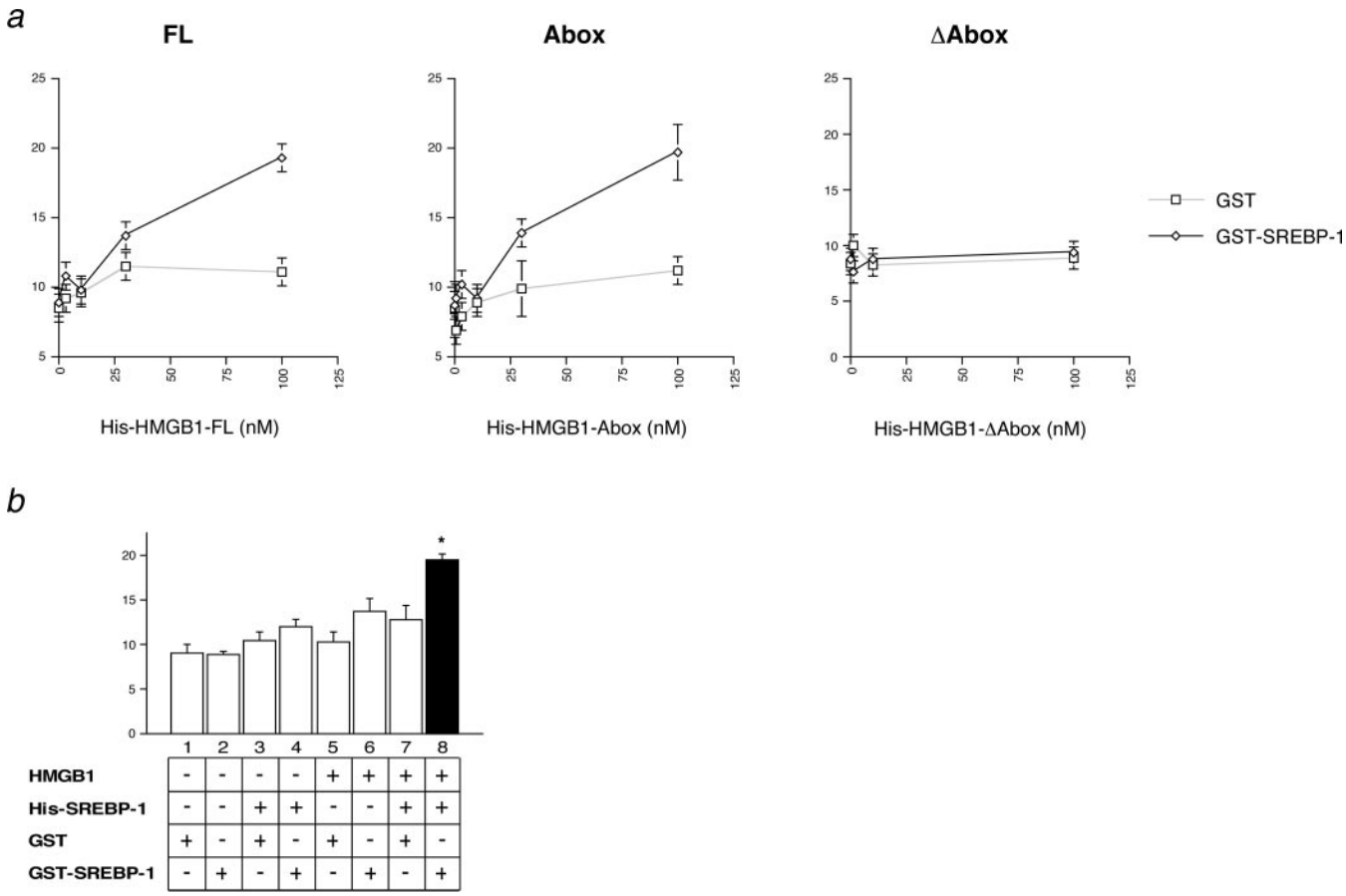


FIG. 9. **TR-FRET assays demonstrating that HMGB1 promotes SREBP-1 dimerization.** *a*, TR-FRET observed between europium and allophycocyanin, which were used to label His-HMGB1 and GST-SREBP-1, respectively, with their conjugated antibody. Full-length HMGB1 (*FL*, *left panel*) and HMGB1 with A box alone (*Abox*, *middle panel*) proteins were shown to provoke FRET signals, whereas HMGB1 lacking A box (Δ*Abox*, *right panel*) was not. *b*, TR-FRET between His-SREBP-1 and GST-SREBP-1 labeled with europium and allophycocyanin, respectively, was measured in the presence or absence of HMGB1 (without any tags). Combination of His-SREBP-1 and GST-SREBP-1 in the presence of HMGB1 (*lane 8*) exhibited significantly higher FRET signals. *, significance at $p < 0.001$ by Fisher's test against any control. Time-resolved fluorescence exited at 340 nm and detected at 665 nm with delay time of 50 μs was measured by cumulative counts of 1000 repeats. The results are expressed as 1000 × (665 nm/615 nm). *Bars*, ± S.E.

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 SREBPs, because SREBPs have been proposed to be also involved in another type of lipodystrophy caused by 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.

Acknowledgments—We appreciate Alyssa H. Hasty for critical reading of the manuscript, Yoshiya Oda and Hiroyuki Arai for technical advice for mass spectrometry, and Kengo Miyagai and Taro Nakazawa for assistance of TR-FRET assay.

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