Insulin-activated Erk-mitogen-activated Protein Kinases Phosphorylate Sterol Regulatory Element-binding Protein-2 at Serine Residues 432 and 455 in Vivo*

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The transcription factor sterol regulatory element binding protein (SREBP)-2 plays a pivotal role in lipid metabolism. Previously, we have shown that the mature form of SREBP-2 is a substrate of Erk-mitogen-activated protein kinases (MAPK). The aim of the present study was to identify Erk-specific phosphorylation sites. Using a protein chemistry approach, we could identify Ser-432 and Ser-455 as major phosphorylation sites. Further characterization by electrophoretic mobility shift assay and promoter reporter gene analyses revealed that phosphorylation does not influence protein/DNA interaction, but enhances trans-activity. In intact cells, SREBP-2 is phosphorylated by insulin, which seems to be related to their bio-responses on low density lipoprotein receptor activity. These results suggest that activation of Erk-MAPK pathways by hormones such as insulin might be related to a novel regulatory principle of SREBP-2.

Sterol regulatory element binding proteins (SREBPs) are a family of basic helix-loop-helix transcription factors that are embedded as precursor proteins in the endoplasmic reticulum and nuclear envelope. To date, three SREBP isoforms have been detected: SREBP-1α, SREBP-1c (shorter splicing variant of SREBP-1a), as well as SREBP-2 (1–5). SREBP-2 is a major regulator of cholesterol homeostasis, whereas SREBP-1c regulates predominantly de novo synthesis of fatty acids. In contrast, SREBP-1a seems to influence both lipogenic and cholesteryl ester genes (6).

Activation of SREBPs initiated by cellular cholesterol depletion is mediated by sequential cleavage (7). As a result, the amino-terminal domain of the protein translocates into the nucleus and activates transcription of target genes. Beside this mechanism, which controls the abundance of activated SREBPs in the cell, we have demonstrated that trans-activity of the N-terminal domain of SREBPs is regulated directly by extra cellular stimuli, e.g. by hormones such as insulin (8, 9). Moreover, in these studies, we have shown that the N-terminal domains of SREBP-1α, SREBP-1c, and SREBP-2 are substrates of the extracellular signal-regulated kinase (Erk) subfamily of mitogen-activated protein kinases (MAPK). In this study, we have identified Ser-432 and Ser-455 as the major phosphorylation sites of Erk-MAPK in SREBP-2 using protein chemistry methodology. This phosphorylation has no influence on DNA interaction but affects trans-activity of SREBP-2. Accordingly, in cells, activation of low density lipoprotein (LDL) receptor gene by insulin is coupled to the identified Erk-specific phosphorylation sites in SREBP-2.

EXPERIMENTAL PROCEDURES

Plasmids and Molecular Cloning—Construction of the LDL receptor (LDLR) promoter reporter gene plasmid containing a functional intact sterol regulatory element (sre)-1 flanked by two SP1 elements (pLDL4-luc) was described previously (10). The N-terminal domain of human SREBP-2 (amino acids 1–468) was inserted in-frame to glutathione S-transferase (GST) into pGEX-3X (Amersham Biosciences) to generate GST-fusion proteins (9). The construction of mutated forms of the N-terminal domain of SREBP-2 (SREBP-2-NT) (S432A, S455A, and S432A/S455A) was performed as described below. Vector for expressing SREBP-2-NT or mutated SREBP-2-NT in mammalian cells was constructed by ligating the N-terminal domain as a BamHI/EcoRI fragment (1403 base pairs) into the BamHI and EcoRI site of pcDNA3.1HisA (Invitrogen). Subsequently, hemagglutinin (HA) tag was added to the 5’ end of SREBP-2-NT constructs after linearization with BamHI by PCR through the deletion of the His tag and first codon of SREBP-2 using the following primers: sense primer (single-underlined indicates the DNA sequence of HA peptide and double-underlined indicates the DNA sequence of yeast transcription factor Gal4 DNA binding domain (amino acids 1–147). Then, for in-frame insertion, the construct was amplified by PCR reaction was digested with DpnI, followed by religation.

To construct Gal4-SREBP-2-NT, Gal4-SREBP-2-NT S432A, Gal4-SREBP-2-NT S455A, and Gal4-SREBP-2-NT S432A/S455A, the corresponding fragments were ligated as a BamHI/EcoRI fragment into the BamHI and EcoRI site of expression vector pEA-CMV (Stratagene) containing the DNA-binding domain of yeast transcription factor Gal4 (amino acids 1–147). Then, for in-frame insertion, the construct was digested with BamHI, refilled with Klenow construct, and religated. The sequences of the constructs were confirmed by DNA sequencing (373A Applied Biosystem Inc.).

Reporter plasmid pG5-Luc containing five Gal4 DNA-binding sites cloned upstream of a minimal promoter element, and the firefly luciferase gene was obtained from Promega. The Renilla expression vector pRL(-mcs) was generated by eliminating the multiple cloning site of pRL-null vector supplied from Promega. Expression vector pUSE/MEK2 for dominant-active MEK2 S222D/S226D under control of cytomegalovirus (CMV) promoter was purchased from Upstate Biotechnology.

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‡ The abbreviations used are: SREBP, sterol regulatory element binding proteins; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinases; sre, sterol regulatory element; GST, glutathione S-transferase; SREBP-2-NT, N-terminal domain of SREBP-2; HA, hemagglutinin; CMV, cytomegalovirus; EMSA, electro-mobility shift assay; HPLC, high performance liquid chromatography; LDLR, LDL receptor; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; SDS, sodium dodecyl sulfate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; bHLH-LZ, basic helix-loop-helix leucine-zipper.
Site-directed Mutagenesis—The Ser → Ala mutants were created by site-directed mutagenesis of pGEX-3X-SREBP-2-NT using the QuickChange Kit (Stratagene) according to the manufacturer’s recommendations. The following primers were used for amplification reaction (complementary 3’ primer is not listed; mutated nucleotides are in bold); Ser-432 → Ala (nt 1411: Thr → Gyl), 5'-GAAATGTCCTTCTT-GATGGCCCCCCAGCTCTCG-3'; Ser-455 → Ala (nt 1480/1481: AG → GGc, 5'-CATTGACTCTGAGCCAGGAGCCCCCTATTGGAAGTGAAAGC-3’.

Fusion Protein Production and Protein Kinase Assay—GST-SREBP-2-NT, GST-SREBP-2-NT S432A, GST-SREBP-2-NT S455A, and GST-SREBP-2-NT S432A/S455A fusion proteins were expressed in Escherichia coli strain BL21-Codon plus (DE3)-RP (Stratagene) and purified according to the manufacturer’s recommendations (Amersham Biosciences). Protein phosphorylation by MAPK Erk1 and Erk2 (Upstate Biotechnology) was performed with 10μg of GST-SREBP-2-NT fusion protein or 10μg of mutated GST-SREBP-2-NT (S432A, S455A, S432A/S455A) fusion protein and activated GST-Erk1 (40ng/μg substrate) or GST-Erk2 fusion protein (10ng/μg substrate) in kinase buffer (25 mM Tris-HCl, pH 7.5, 6.25 mM β-glycerophosphate, 1.25 mM EGTA, 0.25 mM sodium orthovanadate, 0.25 mM dithiothreitol, 10 mM MgCl₂). The reaction was initiated by the addition of 50μM [γ-32P]ATP and 0.1 mCi/ml in a final volume of 40 μl of kinase buffer. The reaction was terminated after 15 min at 25°C by the addition of 5× sodium dodecyl sulfate (SDS) loading buffer and boiling for 5 min. Phosphorylated proteins were separated by SDS-10% PAGE and analyzed by autoradiography of the Coomassie-stained dried gels.

For electro-mobility shift assay (EMSA), 1μg of pcDNA3.1HisA/SREBP-2-NT was in vitro transcribed and translated as His-tagged protein by TNT T7/T3-coupled reticulocyte lysate system, according to the manufacturer’s recommendations, in a final volume of 50 μl (Promega). Thereafter, the His-SREBP-2-NT was purified by using Ni-NTA magnetic agarose beads according to the supplier’s instruction manual (Qiagen). For phosphorylation, 200 ng of isolated protein was incubated with recombinant activated MAPK Erk2 (1 ng/0.1 μg substrate) in kinase buffer (25 mM Tris-HCl, pH 7.5, 6.25 mM β-glycerophosphate, 1.25 mM EGTA, 0.25 mM sodium orthovanadate, 0.25 mM dithiothreitol, 10 mM MgCl₂) under conditions specified in the supplier’s instruction manual (Upstate Biotechnology). The reaction was started by the addition of ATP to a final concentration of 10 μM and was carried out in a final volume of 50 μl for 15 min at 25°C. To control phosphorylation efficiency, kinase reaction was performed with an aliquot of His-SREBP-2-NT using an additional 10 μCi [γ-32P]ATP.

Identification of Phosphorylation Sites by High Performance Liquid Chromatography (HPLC) and Nanoelectrospray Mass Spectrometry—500 μg of GST-SREBP-2-NT fusion protein (7 nmol) was phosphorylated by 50 μg of GST-Erk2 as described above with 250 μM ATP (specific activity 100 cpm/μmol, Cerenkov radiation). The proteins were separated by SDS-10% PAGE, and phosphorylated GST-SREBP-2-NT was digested with 50 μg of trypsin (sequencing grade, Roche Applied Science) in the excised gel pieces overnight at 37°C. The peptides were eluted with 50 mM ammonium carbonate and separated on an anion-exchange column (Nucleoigel SAX 1000–8/46, 50 × 4.6 mm, Machery & Nagel) using a Beckman gold solvent delivery system. The HPLC flow rate was set at 0.5 ml/min. After injection of a 1-ml sample, the peptides were eluted, beginning with 100% buffer A (20 mM NH₄COOH, pH 7.0) and 0% of buffer B (0.5 mM KH₂PO₄, pH 4.0). Ratios of solution B were increased from 0 to 10% in 40 min and from 10 to 50% in 75 min. Fractions of 0.5 ml were collected. Radioactive fractions were subjected to reversed-phase HPLC. These peptides were separated on a C₁₈ reversed-phase column (150 × 0.8 mm, 5-μm particle size, 300-A pore size, Atlantis, Waters, Milford, Massachusetts). The fraction containing SREBP-2-NT were tryptically digested, and the resulting SREBP-2-NT peptides were analyzed by anion-exchange HPLC. Peptides were eluted by a buffer B gradient containing 0.5 mM KH₂PO₄, pH 4. The elution profiles of SREBP-2-NT after Erk1 (A) and Erk2 (B) phosphorylation are shown; the major radioactive peaks of the latter are labeled as D1 and D2 (fractions 45 and 57, respectively). For identification of Erk-MAPK-specific phosphorylation sites, 500 μg of GST-SREBP-2-NT were phosphorylated by Erk2-MAPK (10ng/μg substrate) in vitro and separated by SDS-10% PAGE. The excised radioactive slices containing SREBP-2-NT were trypsin digested and the resulting SREBP-2-NT peptides were separated by anion-exchange HPLC. Fractions corresponding to peak 1 (C) or peak 2 (D) in the anion-exchange HPLC were pooled and subjected to rechromatography by C₁₈ reversed-phase HPLC. Peptides were eluted with a 0–95% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The eluant was monitored by UV absorbance at 214 nm. Radioactive fractions are designated with asterisks. By using electrospray ionization mass spectrometry and tandem mass spectrometry and Edman degradation for peak 1, the peptide HEDPNVLLMS*PPASDSSGQAFSP’y (amino acids 423–447) with phosphorylated Ser-432 and for peak 2, the peptide SIDSEPGPS*PLDDAK (amino acids 448–462) with phosphorylated Ser-455 were identified. Reactions performed are described under “Experimental Procedures.”

FIG. 1. Peptide map analysis of phosphorylated SREBP-2-NT. 10 μg of GST-SREBP-2-NT were separated by SDS-10% PAGE after phosphorylation by activated recombinant MAPK Erk1 (40 ng/μg substrate) and Erk2 (10 ng/μg substrate). The excised radioactive slices containing SREBP-2-NT were trypsinically digested, and the resulting SREBP-2-NT peptides were analyzed by anion-exchange HPLC. Peptides were eluted by a buffer B gradient containing 0.5 mM KH₂PO₄, pH 4. The elution profiles of SREBP-2-NT after Erk1 (A) and Erk2 (B) phosphorylation are shown; the major radioactive peaks of the latter are labeled as D1 and D2 (fractions 45 and 57, respectively). For identification of Erk-MAPK-specific phosphorylation sites, 500 μg of GST-SREBP-2-NT were phosphorylated by Erk2-MAPK (10ng/μg substrate) in vitro and separated by SDS-10% PAGE. The excised radioactive slices containing SREBP-2-NT were trypsinatically digested, and the resulting SREBP-2-NT peptides were separated by anion-exchange HPLC. Fractions corresponding to peak 1 (C) or peak 2 (D) in the anion-exchange HPLC were pooled and subjected to rechromatography by C₁₈ reversed-phase HPLC. Peptides were eluted with a 0–95% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The eluant was monitored by UV absorbance at 214 nm. Radioactive fractions are designated with asterisks. By using electrospray ionization mass spectrometry and tandem mass spectrometry and Edman degradation for peak 1, the peptide HEDPNVLLMS*PPASDSSGQAFSP’y (amino acids 423–447) with phosphorylated Ser-432 and for peak 2, the peptide SIDSEPGPS*PLDDAK (amino acids 448–462) with phosphorylated Ser-455 were identified. Reactions performed are described under “Experimental Procedures.”
FIG. 2. HPLC analysis of phosphorylated wild type and mutated forms of SREBP-2-NT by Erk2-MAPK. A, autoradiography of 10% SDS-polyacrylamide gel electrophoresis of 10 μg of GST-SREBP-2-NT and 10 μg each of mutated GST-SREBP-2-NT (S432A, S455A, as well as S432A/S455A) phosphorylated by activated recombinant Erk2-MAPK. The excised radioactive slices containing phosphorylated GST-SREBP-2-NT (B), GST-SREBP-2-NT S432A (C), GST-SREBP-2-NT S455A (D), or GST-SREBP-2-NT S432A/S455A (E) were digested in-gel with trypsin. Peptides were eluted and analyzed by HPLC anion-exchange chromatography using a buffer B gradient containing 0.5 M KH₂PO₄, pH 4. Reactions performed are described under “Experimental Procedures.”
GATACCCGTGATCGAGAG-3' (12). Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in a solution containing 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA followed by heating to 90 °C for 2 min and then cooling to room temperature. The annealed oligonucleotides were 32P-labeled using a Ready-To-Go T4 polynucleotide kinase kit according to the supplier’s instruction manual (Amersham Biosciences). The binding reactions were carried out in a volume of 20 μl containing 15 mM Hepes/KOH, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.5 μg poly[d(I-C)] (Roche Applied Science) and 10 fmol of 32P-labeled DNA with 10 μl of protein mixture. The samples were incubated at 25 °C for 20 min and immediately electrophoresed on a native 5% (w/v) polyacrylamide gel containing 0.5 × TBE (1 × TBE: 89 mM Tris-boric acid, pH 8.3, 1 mM EDTA). Electrophoresis was performed at 150 V for 1.5 h at 4 °C. EMSA was analyzed by autoradiography of the dried gel. Equal loading was confirmed by Western blot analysis of 20 μl of protein mixture.

**Cell Culture, Transient Transfection, and Dual Luciferase Reporter Gene Assay—**HepG2-cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS) (Invitrogen) and antibiotics (Invitrogen) in a humidified 5% CO2 atmosphere at 37 °C. Before transfection, cells were released by trypsinization, washed with 1× phosphate-buffered saline and resuspended in Opti-MEM (Invitrogen) supplemented with 10% (v/v) FCS. For the reporter gene assay, cell suspension (2 × 10^6 cells/well) was mixed with reporter plasmid pL342NT-luc (0.5 μg/well), with Renilla expression vector pRL-mcs (0.1 μg/well) for controlling transfection efficiency, and either with pcDNA3/HA-SREBP-2-NT, pcDNA3/HA-SREBP-2-NT S432A, pcDNA3/HA-SREBP-2-NT S455A, pcDNA3/HA-SREBP-2-NT S432A/S455A, or pcDNA3.0 vector (25 ng/well) as indicated in the figure legends. For testing trans-activity of SREBP-2 and mutants, cell suspension (2 × 10^6 cells/well) was mixed with reporter plasmid pG5-luc (0.5 μg/well), with Renilla expression vector pRL-mcs (0.1 μg/well) for controlling transfection efficiency, and either with pFA/Gal4-SREBP-2-NT, pFA/Gal4-SREBP-2-NT S432A, pFA/Gal4-SREBP-2-NT S455A, pFA/Gal4-SREBP-2-NT S432A/S455A, or pFA-CMV vector (25 ng/well), and with pUSE/MEK2D or pUSE vector (25 ng/well) as indicated in the figure legends. Thereafter, samples were transferred to an electroporation cuvette (inter-electrode distance: 0.4 cm, Bio-Rad) and pulsated at 18 msec in GenePulsor II (Bio-Rad). Before seeding on six-well plates (Greiner Bio-One), cell suspension was diluted with RPMI 1640 medium supplemented with 10% (v/v) FCS and antibiotics. On day 1 after transfection, cells were cultured in RPMI 1640 medium containing 0.5% lipoprotein-deficient serum (LPDS) (Sigma) for 16 h. Subsequently, cells were harvested in 100 μl of passive lysis buffer (supplied with the dual luciferase reporter assay system (Promega); firefly as well as Renilla luciferase activities of cell extracts (20 μl/probe) were measured according to the supplier’s instructions (Promega) with a microplate luminometer LB96V (EG&G Berthold).

To verify the relevance of the identified phosphorylation sites in SREBP-2 for insulin-mediated signaling in the cellular context, cell suspension (2 × 10^6) was mixed with 5 μg of pcDNA3/HA-SREBP-2-NT or mutated SREBP-2-NT constructs and transiently transfected by electroporation (18 msec). Subsequently, cells were diluted with RPMI 1640 medium supplemented with 10% (v/v) FCS and antibiotics and seeded in 35-mm dishes (1 × 10^6/dish). For treatment, cells were cultured in RPMI 1640 medium containing 0.5% LPDS on day 1 after transfection for 16 h. Then, cells were incubated for 45 min without or with insulin (1 × 10^{-5}), and whole-cell extracts were analyzed by Western blotting.

**Preparation of Cell Extracts—**Whole-cell extracts were prepared by scraping off the cells in 250 μl of radiimmune precipitation assay buffer (50 mM Tris-phosphate-buffered saline, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) on culture dishes. The suspensions were incubated on a thermomixer (Eppendorf) for 30 min at 4 °C. Subsequently, lysates were centrifuged at 16,000 × g for 30 min, and aliquots of supernatants were frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were measured using a Bio-Rad protein assay.

**Western Blot Analysis—**Cell extracts were mixed with 5× SDS loading buffer and resolved on a 5.5% SDS-polyacrylamide gel (59:1, acrylamide: bisacrylamide). To obtain better separation of the phosphorylated form of SREBP-2-NT from the unphosphorylated SREBP-2-NT, 4 mM urea was added to the separation gel. EMSA samples were mixed with 5 μl of SDS loading buffer and separated on an SDS-7.5% PAGE. Subsequently, proteins were electroblotted to nitrocellulose membrane (Hybond™ ECL, Amersham Biosciences). After blocking overnight at 4 °C in 1% (w/v) blocking solution (Roche Applied Science) in 1× Tris-buffered saline, pH 7.4, membrane with proteins of cell extracts was probed with rat monoclonal antibody against HA peptide (YPYDVPDYA) conjugated with peroxidase (clone 3F10, 1:5000, Roche Applied Science). Membrane with EMSA samples was probed with antibody directed against His-tag (anti-His-HRP, 1:1000, Invitrogen). Visualization was performed with ECL™ plus Western blotting detection reagents according to manufacturer’s instructions (Amersham Biosciences) and Kodak X-Omat AR film.

**RESULTS**

Previously, we have shown that the N-terminal domain of SREBP-2 (SREBP-2-NT) is a substrate of Erk2 (6) (ERK2 in vitro) (9). Phospho-amino acid analyses revealed that phosphorylation of SREBP-2-NT by MAPK with or without affinity chromatography on an SDS-7.5% PAGE. Subsequently, proteins were electroblotted to nitrocellulose membrane (Hybond™ ECL, Amersham Biosciences). After blocking overnight at 4 °C in 1% (w/v) blocking solution (Roche Applied Science) in 1× Tris-buffered saline, pH 7.4, membrane with proteins of cell extracts was probed with rat monoclonal antibody against HA peptide (YPYDVPDYA) conjugated with peroxidase (clone 3F10, 1:5000, Roche Applied Science). Membrane with EMSA samples was probed with antibody directed against His-tag (anti-His-HRP, 1:1000, Invitrogen). Visualization was performed with ECL™ plus Western blotting detection reagents according to manufacturer’s instructions (Amersham Biosciences) and Kodak X-Omat AR film.

**Peptide Map Analysis by HPLC of in Vitro Phosphorylated SREBP-2-NT**—Erk1 and Erk2 are closely related MAPK; therefore, we tested whether both target the same phosphorylation sites in SREBP-2-NT. The N-terminal domain of GST-
SREBP-2 was incubated with recombinant GST-Erk1 as well as GST-Erk2 in the presence of radioactive $[^{32}P]ATP$ in vitro. The reaction was stopped after 15 min by the addition of loading buffer, proteins were separated by SDS-10% PAGE, and dried gel was exposed to x-ray film. Subsequently, the specific radioactive bands of the proteins were trypic-digested, and eluted peptides were separated by anion-exchange HPLC. The elution profile of Erk1-phosphorylated SREBP-2-NT revealed two major peaks (fractions 45 and 57) in addition to several minor peaks (Fig. 1A). Peak distribution in the elution profile of SREBP-2-NT-phosphorylated Erk2 was identical (Fig. 1B).

Identification of Ser-432 and Ser-455 as Erk-MAPK-specific Phosphorylation Sites in SREBP-2-NT—To separate co-migrating peptides, radioactive fractions of Erk2-phosphorylated SREBP-2-NT corresponding to peak 1 or peak 2 of anion-exchange HPLC (see Fig. 1B) were pooled and analyzed by C$_{18}$ reversed-phase HPLC. The spectra revealed two phospho peptides for peak 1 (Fig. 1C, see asterisks) and only one phosphorylated peptide for peak 2 (Fig. 1D). Further analyses using electrospray ionization mass spectrometry and tandem mass spectrometry led to the identification of Ser-432 and Ser-455 as phosphorylated amino acids in the phosphopeptides IEDFNQNVLLM$^{*}$PPASDSGSQAGFSPY (amino acids 423-447) (peak 1) and SIDSEPG$^{*}$PLLDDAK (amino acids 448-462) (peak 2), respectively. Fraction 19 of peak 1 revealed no analyzable results.

Verification of Ser-432 and Ser-455 as Major Phosphorylation Sites in SREBP-2—To verify the identified phosphorylation sites in SREBP-2-NT, Ser-432 and Ser-455 were replaced by alanine using site-directed mutagenesis. Wild type, single- and double-mutated SREBP-2-NTs (S432A, S455A, and S432A/S455A) were expressed as GST fusion proteins, purified, and incubated with GST-Erk2 in the presence of radioactive $[^{32}P]ATP$ in vitro. The reaction was stopped after 15 min, the protein mix was separated by SDS-PAGE, and the gel was exposed to x-ray film (Fig. 2A). Autoradiography indicated that mutation of Ser-432 and Ser-455 to alanine reduced phosphate incorporation by 40 and 50%, respectively. Phosphate incorporation of the double-mutant S432A/S455A was even reduced by $\sim$70%. Phosphorylated fusion protein bands were cut out, gel slices were incubated with trypsin, and derived peptides were

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**Fig. 4.** Role of identified phosphorylation sites for trans-activity. A, diagram of the different Gal4 DNA-binding domain-SREBP-2-NT fusion proteins used for transient expression assays. The pG5-luc promoter contains 5 copies of the Gal4 element inserted upstream of the minimal promoter (TATAA). The luciferase gene driven by the Gal4 promoter was used as reporter. B, HepG2 cells were transiently transfected with pG5-luc (0.5 μg/well), pRL-mcs (0.1 μg/well), and SREBP-2-NT or the mutants S432A, S455A, S432A/S455A in pFA-CMV (25 ng/well) and with 25 ng/well pUSE-mock (dark gray bars) or 25 ng/well pUSE-MEK2 (hatched bars), as described under “Experimental Procedures.” The promoter strength is represented by the absolute luciferase activity normalized to renilla activity measured in the cellular extract. Results are given as mean ± S.D. of five independent experiments, each performed in triplicate.
analyzed by anion-exchange chromatography. In contrast to the elution profile of SREBP-2-NT (Fig. 2B), peak 1 was missed in the profile of tryptic-digested SREBP-2-NT S432A (Fig. 2C), and peak 2 disappeared when Ser-455 was mutated to alanine (Fig. 2D). Consistently, the elution profile of the double-mutant S432A/S455A lacked both major peaks (Fig. 2E).

Functional Relevance of Phosphorylation in SREBP-2-NT for Protein/DNA Binding and Trans-activity—Phosphorylation can influence protein/DNA interaction or trans-activity of transcription factors (13). To analyze protein/DNA interaction, in vitro transcribed and translated His-SREBP-2-NT was incubated without or with Erk2-MAPK and subsequently analyzed by EMSA. Equal loading was verified by Western blot analysis, and phosphorylation efficiency was controlled by using radio-labeled ATP for kinase reaction in separate assays (Fig. 3, A and B). Autoradiography of EMSA showed that SREBP-2-NT interacted with DNA-fragments containing sre-1 (Fig. 3C) as well as E-box motive (Fig. 3D) to a similar degree, and phosphorylation of SREBP-2-NT (lane 8) had no effect upon protein/DNA interaction with either motive (compare lane 7 with lane 8 in Fig. 3).

To test the influence of phosphorylation on trans-activity, we performed promoter reporter gene assays recruiting the PathDetect trans-reporting system (Stratagene). In this assay, SREBP-2-NT, SREBP-2-NT S432A, SREBP-2-NT S455A, and SREBP-2-NT S432A/S455A were expressed as fusion proteins with the DNA-binding domain of yeast Gal4 (amino acids 1–147) (Fig. 4). The DNA-binding domain alone does not have any trans-activity per se. Accordingly, detected activity reflects trans-activity of the fused domain, i.e. SREBP-2-NT or mutants. HepG2 cells were transfected with Gal4-SREBP-2-NT, single and double mutants, and with upstream activator of Erk-MAPK cascade MEK2D for testing the effect of Erk in vivo. The basal trans-activity of Gal4-SREBP-2-NT was 83,967.4 ± 14,595.06 relative light units, after cotransfection with MEK2D SREBP-2-NT trans-activity was elevated ~3.5-fold. Basal activities of SREBP-2-NT S432A, SREBP-2-NT S455A, as well as SREBP-2-NT S432A/S455A were comparable with SREBP-2-NT. On the contrary, cotransfection with MEK2D increased trans-activity of SREBP-2-NT S432A just 1.6-fold and trans-activity of SREBP-2-NT S455A 1.4-fold. In the case of the double-mutant SREBP-2-NT S432A/S455A, no stimulation occurred.

Functional Relevance of Phosphorylation in Vivo—To test the relevance of identified phosphorylation sites in a cellular context, HepG2 cells were transfected with SREBP-2-NT or mutated forms of SREBP-2-NT and incubated with insulin for 45 min (Fig. 5). Western blot analysis of prepared cell extracts revealed that wild type-related phosphorylation was significantly reduced in single-mutated SREBP-2-NT and completely abolished in the double-mutant. Previously, we have shown that the stimulatory effect of insulin on LDL receptor promoter is mediated by SREBPs (9, 10). To prove that the insulin-induced activation of LDL receptor promoter is coupled to the identified phosphorylation sites in SREBP-2, we performed promoter reporter gene assays. HepG2 cells were transiently transfected with LDL receptor promoter construct plL4-luc, HA-SREBP-2-NT, or mutated SREBP-2-NTs. On day 1 after transfection, cells were incubated with insulin for 3 h, and after cell lysis, reporter gene activity was measured. Fig. 6 shows...
that basal activity of LDL receptor promoter (310.23 ± 108.78 relative light units) was strongly increased by ectopic expression of wild-type SREBP-2-NT (6527.11 ± 2379.48 relative light units). Incubation with insulin elevated the promoter activity in a synergistic fashion. Basal activities of SREBP-2-NT S432A, SREBP-2-NT S455A, as well as SREBP-2-NT S432A/S455A were comparable with SREBP-2-NT. On the contrary, in cells, ectopically expressed SREBP-2-NT S432A incubation with insulin increased promoter activity just 1.3-fold and 1.4-fold, respectively; ectopically expressed SREBP-2-NT S455A incubation with insulin increased promoter activity just 1.2-fold and 1.3-fold, respectively. In the case of the double-mutant SREBP-2-NT S432A/S455A, basal activity was comparable with wild type, but no stimulation occurred by insulin. These results show that the synergistic activation of SREBP-2 by insulin is dependent upon phosphorylation.

DISCUSSION

Cell surface events lead to modification of cell-specific gene expression patterns, e.g. modulating signal transduction pathways (14). Recruitment of the MAPK cascades is a general mechanism for a rapid gene regulatory answer of cells to environmental stimuli by setting the activation state of different transcription factors. The core module of all MAPK cascades consists of three kinases, which are activated by sequential phosphorylation. To date, several MAPK cascades have been defined; in mammalian cells, the best characterized are the Erk, the stress-activated cJun N-terminal kinases and the cytokine-suppressive anti-inflammatory drug-binding p38 kinase pathways (15). These highly conserved proline-directed serine/threonine kinases are involved in hormone action, apoptosis, cell growth, and differentiation. Previously, we demonstrated that the SREBP transcription factors are targets of intracellular signaling pathways and substrates of Erk1 and Erk2 (8, 9). Furthermore, in SREBP-1a, we identified Ser-117 as a major Erk-MAPK phosphorylation site (16). By using the same protein chemistry approach, we have identified in this study serine residues 432 and 455 as major phosphorylation sites of Erk in SREBP-2-NT

Structural features of the transcription factors SREBPs show that they belong to the basic helix-loop-helix leucine zipper (bHLH-LZ) super-family (17, 18). Most bHLH-LZ proteins bind as either homo- or hetero-dimers to a consensus DNA sequence. DNA binding is mediated by a region of basic amino acids immediately upstream to the bHLH-LZ dimerization domain. In addition to the DNA-binding domain and the protein-binding domain on the C-terminal side, there is an acidic domain in the SREBP N-terminal region, which seems to play a role in trans-activity (19, 20). The identified Erk-MAPK phosphorylation site Ser-117 in SREBP-1a is located next to the N-terminal acidic domain of SREBP-1a (amino acids 1–61). Trans-activity of SREBP-1a in response to insulin stimulation of Erk-MAPK phosphorylation seems to be due to phosphorylation-mediated modifications of this region. In contrast, both SREBP-2-NT phosphorylation sites Ser-432 and Ser-455 are near the bHLH-LZ (amino acids 331–401) domain, which may affect dimerization and DNA binding.

Thus, phosphorylation of one or both sites in SREBP-2-NT may influence the generation of homo- as well as hetero-dimers, a phenomenon that has been shown for the transcription factor Max. CKII-mediated phosphorylation of Max prevents Max homo-dimerization, whereas formation of Myc-Max hetero-dimers is not affected (21–23). Another case is the influence of phosphorylation on protein/DNA-binding interaction of many transcription factors, e.g. ER81 or TAL1/SCL. For the basic helix-loop-helix transcription factor TAL1/SCL, it has been shown that phosphorylation at Ser-172, which is located in a region proximal to the DNA-binding domain, alters DNA binding without interference with the protein/protein interaction, whereas phosphorylation of Ser-122 elevates trans-activity (24, 25). On the other hand, DNA-binding ability of the ETS transcription factor ER81 is reduced and trans-activity is enhanced by phosphorylation on Ser-334 (26). To test the effect of phosphorylation on DNA binding of SREBP-2-NT, we performed EMSA. Because of his dual binding specificity to two distinct DNA motifs, i.e. both sre-1 and E-box (12), we analyzed whether the binding ability to one of them was influenced by phosphorylation. Our investigations show no influence on both binding motifs, implying that DNA-binding ability and at least dimerization of SREBP-2-NT is not affected by phosphorylation.

In addition to DNA interaction, phosphorylation could influence trans-activity. Using a heterologous promoter reporter gene system reveals that mutation of the phosphorylation sites in SREBP-2-NT did not affect basal activity, but dramatically reduces inducibility by MEK2D. This result demonstrates that Erk2-dependent phosphorylation plays a major role in the regulation of SREBP-2-NT trans-activity, and that this transcription factor is a relevant substrate of the Erk-MAPK cascade.

Taken together, we have shown that SREBP-2 is phosphorylated by Erk MAPK at Ser-432 and Ser-455. Moreover, these sites are not only targets for Erk1 or Erk2 in vitro but also in intact cells. Furthermore, direct evidence is provided that SREBP-2 is phosphorylated by stimulating cells with insulin, and that identified phosphorylation sites seem to play a role in mediating the effects of insulin on LDLR promoter activity. In support of this idea, we have previously shown that the effects of both inducers of LDLR promoter activity are abolished by inhibiting the Erk-MAPK pathway in cells lacking SREBP-2 (9). At the least, these data support our concept that, besides sterol-dependent cleavage, SREBPs are regulated by phosphorylation affecting their trans-activity. Therefore, cells are able to react more individually upon environmental stimuli.

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