Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors that regulate cholesterol and lipid metabolism. The active forms of these transcription factors are targeted by a number of post-translational modifications, including phosphorylation. Phosphorylation of Thr-426 and Ser-430 in SREBP1a creates a docking site for the ubiquitin ligase Fbw7, resulting in the degradation of the transcription factor. Here, we identify a novel phosphorylation site in SREBP1a, Ser-434, which regulates the Fbw7-dependent degradation of SREBP1. We demonstrate that both SREBP1a and SREBP1c are phosphorylated on this residue (Ser-410 in SREBP1c). Importantly, we demonstrate that the mature form of endogenous SREBP1 is phosphorylated on Ser-434. Glycogen synthase kinase-3 phosphorylates Ser-434, and the phosphorylation of this residue is attenuated in response to insulin signaling. Interestingly, phosphorylation of Ser-434 promotes the glycogen synthase kinase-3-dependent phosphorylation of Thr-426 and Ser-430 and destabilizes SREBP1. Consequently, mutation of Ser-434 blocks the interaction between SREBP1a and Fbw7 and attenuates Fbw7-dependent degradation of SREBP1a. Importantly, insulin fails to enhance the levels of mature SREBP1 in cells lacking Fbw7. Thus, the degradation of mature SREBP1 is controlled by cross-talk between multiple phosphorylated residues in its C-terminal domain and the phosphorylation of Ser-434 could function as a molecular switch to control these processes.

The sterol regulatory element-binding protein (SREBP) family of transcription factors controls cholesterol and lipid metabolism and plays critical roles during adipocyte differentiation and insulin signaling (1–3). The family consists of three different SREBP proteins, SREBP1a, SREBP1c, and SREBP2. The SREBPs are synthesized as transcriptionally inactive precursor proteins that are inserted into the membranes of the endoplasmic reticulum (4). In sterol-depleted cells, SREBPs are processed sequentially by two membrane-associated proteases that release the mature forms of the proteins (5, 6). The mature forms of the SREBPs are translocated to the nucleus where they bind to the promoters of SREBP target genes and regulate expression of the corresponding genes (7). It has been demonstrated that the mature forms of SREBPs are modified by phosphorylation (3, 8–12), acetylation (13), sumoylation (14), and ubiquitination (9, 12, 15, 16). These modifications regulate the stability and/or transcriptional activity of the active transcription factors.

Many transcription factors are unstable proteins that are targeted for degradation by the ubiquitin-proteasome system (17, 18). The mature forms of SREBPs are degraded by the proteasome in an ubiquitin-dependent manner (9, 16). It has been suggested that nuclear SREBP molecules are, at least in part, ubiquitinated and degraded as a functional consequence of their transcriptional activity (12, 15). GSK-3-mediated phosphorylation of Thr-426 and Ser-430 in the C terminus of mature SREBP1 creates a docking site for Fbw7 (also known as human CDC4), the substrate recognition component of a specific SCF ubiquitin ligase (9). Fbw7 is a tumor suppressor (19) and targets cyclin E (20, 21), c-Myc (22, 23), Notch (24–27), and SRC-3 (30) for degradation in a phosphorylation-dependent manner. Fbw7 interacts with nuclear SREBP1 and enhances its ubiquitination and degradation in a manner dependent on the phosphorylation of Thr-426 and Ser-430. The phosphorylation of these two residues is enhanced in response to DNA binding and GSK-3β is recruited to the promoters of SREBP target genes in vivo (12). Consequently, Fbw7 is recruited to SREBP target promoters and induces the ubiquitination and degradation of SREBP1. In the current study, we identify a novel serine residue, Ser-434, in the C terminus of mature SREBP1 that is phosphorylated by GSK-3. Our results demonstrate that the phosphorylation of Ser-434 in SREBP1 is required for the GSK-3-dependent phosphorylation of Thr-426 and Ser-430 in its phosphodegron. Consequently, the phosphorylation of Ser-434 regulates the interaction between SREBP1 and Fbw7, thereby controlling the degradation of mature SREBP1. The phosphorylation of Ser-434 is also enhanced in response to
Phosphorylation-dependent Degradation of SREBP1

DNA binding, and mutation of Ser-434 blocks the phosphorylation of Thr-426 and Ser-430 during this process, suggesting that the phosphorylation of Ser-434 could control the degradation of active SREBP1 molecules. Thus, the degradation of mature SREBP1 is controlled by cross-talk between multiple phosphorylated residues in its C-terminal domain, and the phosphorylation of Ser-434 could function as a molecular switch to control these processes.

EXPERIMENTAL PROCEDURES

Cell Culture—All tissue culture media and antibiotics were obtained from Invitrogen and Sigma. HEK293, HEK293T, HepG2, U2OS, and HeLa cells were from ATCC. Fbw7-positive and Fbw7-negative HCT116 cells were provided by B. Vogelstein (31).

Reagents and Antibodies—Anti-FLAG antibody (M5), cycloheximide, and standard chemicals were from Sigma. Monoclonal anti-Myc (9E10), anti-SREBP1 (2A4), antitubulin (TU-02), anti-GST (B-14), rabbit anti-SREBP1 (H-160), and anti-phosphorylated (Ser-9/Ser-21) GSK-3 (sc-11757) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GSK-3α/β was from Cell Signaling, and recombinant GSK-3β was from New England Biolabs.

Generation of Phosphorylation-specific SREBP1 Antibodies—Synthetic phosphopeptides corresponding to residues 431–437 (Ser-434 phosphorylated) in human SREBP1a were coupled to keyhole limpet hemocyanin before being injected into rabbits. The phosphopeptides and the corresponding nonphosphorylated peptides, as well as phospho-Ser, were coupled to Sulfolink (Pierce) and used as affinity matrices to purify the antibodies from rabbit sera (9, 11).

Plasmids and Transfections—The expression vectors for FLAG-SREBP1a and SREBP1c (amino acid residues 2–490 and 2–466, respectively) have been described (15). Point mutants were generated by site-directed mutagenesis (QuikChange, Stratagene). The HMG-CoA synthase (SYNSRE-luc), LDL receptor (LDLR-luc), and fatty acid synthase (FAS-luc) promoter-reporter constructs have been described (32). All other expression vectors have been described previously (20, 23). Transient transfections were performed using the MBS transfection kit (Stratagene). The Fbw7, GSK-3α/β, and control siRNA were from Ambion and have been described before (18, 35).

Immunoprecipitations and Immunoblotting—Cells were lysed in buffer A (50 mM HEPEs (pH 7.2), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate, 1% aprotinin, 0.1% SDS, and 0.5% sodium deoxycholate) and cleared by centrifugation. For co-immunoprecipitations, cell lysates were prepared in the absence of SDS and sodium deoxycholate. Cell lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). To ensure that equal amounts of protein were loaded in each well, the levels of α-tubulin in the samples were estimated by Western blotting.

Determination of Protein Half-life—Cells were treated with cycloheximide to stop protein synthesis and incubated for the indicated times. Total cell lysates were prepared, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. SREBP1 was visualized by Western blotting followed by quantitation on a charge-coupled device camera (Fujii) and image analysis software (Aida Image Analyzer 3.10).

Luciferase and β-Galactosidase Assays—Cells were transiently transfected with the indicated promoter-reporter genes in the absence or presence of expression vectors for SREBP1, either wild-type or the indicated mutants. After 36 h, luciferase activities were determined in duplicate samples as described by the manufacturer (Promega, Madison, WI). The pCH110 vector encoding the β-galactosidase gene under the control of the SV40 promoter (Amersham Biosciences) was used as an internal control for transfection efficiency. Luciferase values (relative light units) were calculated by dividing the luciferase activity by the β-galactosidase activity. The data represent the average ± S.D. of three independent experiments performed in duplicates.

Reverse Transcription-PCR Assays—RNA was extracted with TRIzol reagent (Invitrogen). Total RNA was subjected to reverse transcription with oligo(dT), followed by PCR with target-specific primers. The PCR reactions, using Invitrogen High Fidelity DNA polymerase, were optimized for the individual target genes. The PCR programs and primer sequences for the human LDL receptor, HMG-CoA synthase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase genes are available on request.

RESULTS

GSK-3 Phosphorylates Ser-434 in SREBP1—We recently identified two phosphorylated residues in the C terminus of mature SREBP1a, Thr-426 and Ser-430 (9). GSK-3 is involved in the phosphorylation of both Thr-426 and Ser-430. However, kinase assays with recombinant GSK-3β and SREBP1a followed by phosphopeptide mapping indicated that the C terminus of mature SREBP1a contained additional residues phosphorylated by GSK-3 (data not shown). The C-terminal domains of mature SREBP1a and SREBP1c are identical. Data base searches suggested that Ser-434 in SREBP1a (Ser-410 in SREBP1c) could be phosphorylated by GSK-3. To determine if SREBP1a was phosphorylated on Ser-434, we generated a phospho-Ser-434-specific anti-SREBP1 antibody (pS434). This antibody recognized wild-type SREBP1a following expression in HEK293T cells, whereas it failed to recognize the S434A mutant (Fig. 1A). Wild-type SREBP1c was also recognized by the antibody, whereas the S410A mutant was not (Fig. 1A), demonstrating that both isoforms of SREBP1 are phosphorylated on this serine residue. In addition, the recognition of wild-type SREBP1a was competed with a peptide containing phospho-Ser-434, but not with peptides in which Thr-426 or Ser-430 was phosphorylated (supplemental Fig. S1), supporting the specificity of the antibody.

Our phosphopeptide mapping suggested that Ser-434 could be phosphorylated by GSK-3β in vitro. Indeed, in vitro kinase assays demonstrated that GSK-3β phosphorylates Ser-434 in SREBP1a (Fig. 1B). To test whether endogenous GSK-3 could phosphorylate Ser-434 in endogenous SREBP1, HeLa cells were treated with lithium, a pharmacologic GSK-3 inhibitor. The

5886 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 284 • NUMBER 9 • FEBRUARY 27, 2009
Phosphorylation of Ser-434 Destabilizes SREBP1—We have previously demonstrated that the mature form of SREBP1 is destabilized following GSK-3-dependent phosphorylation of Thr-426 and Ser-430 (9). To determine if phosphorylation of Ser-434 could be involved in regulating the stability of SREBP1, cells were transfected with mature SREBP1a, either wild-type
Phosphorylation-dependent Degradation of SREBP1

or the phosphorylation-deficient S434A mutant. The steady-state levels of the S434A mutant were enhanced compared with the wild-type protein (Fig. 3A), indicating that phosphorylation of Ser-434 destabilizes SREBP1. Similar results were obtained when the corresponding residue (Ser-410) was mutated in SREBP1c (Fig. 3A), suggesting that phosphorylation of this residue regulates the stability of both SREBP1 isoforms. The S434A mutant of SREBP1a was also stabilized in HEK293, HeLa, and HepG2 cells (supplemental Fig. S3). Interestingly, the steady-state levels of a phosphorylation-mimetic mutant of SREBP1a (S434D) were slightly reduced compared with the wild-type protein (Fig. 3A), further supporting the notion that phosphorylation of Ser-434 destabilizes SREBP1. The mRNA levels of the constructs in Fig. 3 (A and B) were similar (supplemental Fig. S4), suggesting that the expression of these mutants was affected at the protein level. To confirm that the enhanced abundance of the S434A mutant resulted from reduced degradation, we measured the half-life of mature SREBP1a, either wild-type or the S434A mutant, in transfected HEK293T cells. As illustrated in Fig. 3C, the turnover of the mutant protein was reduced compared with wild-type SREBP1a.

The accumulation of the S434A mutant should lead to an enhanced expression of SREBP target genes. To test this hypothesis, HepG2 cells were transfected with an SREBP-responsive promoter-reporter gene in the absence or presence of mature SREBP1a, either wild-type, S434A, or S434D. In support of our hypothesis, the transcriptional activity of the S434A mutant was enhanced, whereas the activity of the S434D mutant was reduced compared with the wild-type protein (Fig. 3D). Mutation of the corresponding residue in SREBP1c (S410) also enhanced its transcriptional activity (Fig. 3D), indicating that the phosphorylation of Ser-434 and its effect on the stability of SREBP1 influences the biological function of SREBP1. This possibility was supported when we analyzed the expression of endogenous target genes in HEK293 cells transfected with mature SREBP1a. The expression of both the LDL receptor and HMG-CoA synthase genes was higher in cells expressing the S434A mutant compared with cells expressing wild-type SREBP1a (Fig. 3E).

If our hypothesis was correct, activation of GSK-3 should induce the degradation of mature SREBP1. To test this, cells were treated with wortmannin and LY294002, two phosphatidylinositol 3-kinase (PI3K) inhibitors. Both inhibitors reduced the inhibitory phosphorylation of GSK-3 and resulted in reduced steady-state levels of mature SREBP1 (Fig. 4A). This effect was specific for the PI3K inhibitors, because the mTor inhibitor rapamycin failed to affect SREBP1 levels. Interestingly, both PI3K inhibitors failed to affect the levels of mature SREBP1 in the presence the proteasome inhibitor MG-132 (Fig. 4B), suggesting that activation of GSK-3 results in enhanced degradation of mature SREBP1. To test this hypothesis, HepG2 cells were transfected with mature SREBP1a, either wild-type, S434A, or the double mutant T426A/S430A and treated with LY294002. As seen in Fig. 4C, LY294002 reduced the levels of wild-type SREBP1a, whereas the degradation of the S434A mutant was attenuated and the double mutant was resistant to LY294002 treatment, suggesting that phosphorylation of Ser-434 and the phosphodegron in SREBP1 is important for this effect. As expected, inhibition of PI3K in HepG2 cells attenuated the accumulation of mature SREBP1 in response to a short pulse of insulin (Fig. 4D). Again, this effect was specific for PI3K, because rapamycin only had a marginal effect, suggesting that PI3K signaling, but not mTor, is important for the acute response to insulin signaling in these cells.

Ser-434 Regulates the Phosphorylation of Thr-426 and Ser-430—Our data indicate that phosphorylation of Ser-434 promotes the degradation of SREBP1. The active form of SREBP1 is targeted for degradation following phosphorylation of Thr-426 and Ser-430. One possibility was therefore that the phosphorylation of Ser-434 could influence the subsequent phosphorylation of Thr-426 and Ser-430. To test this possibility, HEK293T cells were transfected with mature SREBP1a, either wild-type or the T426A, S430A, or S434A mutants, and the phosphorylation ofThr-426, Ser-430, and Ser-434 was measured. As illustrated in Fig. 5A, all three residues were phosphorylated when wild-type SREBP1a was expressed in cells. Muta-
tion of Thr-426 did not only block the phosphorylation of this residue but also significantly reduced the phosphorylation of Ser-430, whereas the phosphorylation of Ser-434 was only somewhat reduced in the T426A mutant. Mutation of Ser-430 attenuated the phosphorylation of Thr-426 and Ser-434. Importantly, mutation of Ser-434 drastically reduced the phosphorylation of Ser-430 and also resulted in a significant attenuation of the phosphorylation of Thr-426. Thus, both Thr-426 and Ser-434 regulate the phosphorylation of Ser-430, and Ser-434 regulates the phosphorylation of both Thr-426 and Ser-430. Therefore, phosphorylation of Ser-434 could potentially regulate the phosphorylation of the phosphodegron in SREBP1, thereby affecting the stability of mature SREBP1. This possibility is in agreement with our observation that the S434A mutant of SREBP1 is stabilized (Fig. 3, A–C).

The results in Fig. 5A suggest that Ser-434 regulates the GSK-3-dependent phosphorylation of Thr-426 and Ser-430. To test this possibility, we used recombinant SREBP1, either wild-type or the S434A mutant, in kinase assays with recombinant GSK-3β. As seen in Fig. 5B, the GSK-3β-dependent phosphorylation of Thr-426 was significantly reduced, and the phosphorylation of Ser-430 completely lost in the S434A mutant, confirming that Ser-434 plays an important role in the phosphorylation of both these residues. Interestingly, mutation of Ser-430 reduced the GSK-3-dependent phosphorylation of Thr-426 (Fig. 5C), suggesting that Ser-430 is a priming site for the phosphorylation of Thr-426. SREBP1 has a serine residue (Ser-438 in SREBP1a) four residues downstream of Ser-434 that could function as a priming site for the phosphorylation of Ser-434. However, mutation of this residue (S438A) failed to affect the phosphorylation of Ser-434, Ser-430, or Thr-426 and failed to affect the steady-state levels of transfected mature SREBP1a (supplemental Fig. S5). Taken together, our results suggest that the GSK-3-dependent phosphorylation of Thr-426 is dependent on the phosphorylation of Ser-430, which in turn is dependent on the phosphorylation of Ser-434.

We recently found that GSK-3β is recruited to SREBP target genes and that the GSK-3β-mediated phosphorylation of Thr-426 and Ser-430 in SREBP1 is enhanced in response to DNA binding (12). To determine if the phosphorylation of Ser-434 is enhanced in response to DNA binding, we performed in vitro kinase assays using recombinant mature SREBP1a and HeLa nuclear extracts in the absence and presence of a promoter fragment containing SREBP binding sites. Similar to Thr-426 and Ser-430, the phosphorylation of Ser-434 was enhanced in response to DNA binding (Fig. 5D). Interestingly, the DNA
Phosphorylation-dependent Degradation of SREBP1

A

FIGURE 4. Activation of GSK-3 induces the degradation of mature SREBP1. A, HepG2 cells were treated for 2 h in the absence or presence of wortmannin (1 \( \mu \)M), LY294002 (5 \( \mu \)M), and rapamycin (50 nM). The expression of SREBP1, GSK-3\( \alpha/\beta \), and \( \alpha \)-tubulin and the phosphorylation of GSK-3\( \alpha/\beta \) were determined by Western blotting. B, HepG2 cells were treated for 2 h with MG-132 (25 \( \mu \)M) in the absence or presence of wortmannin (1 \( \mu \)M) and LY294002 (5 \( \mu \)M). The expression of SREBP1, GSK-3\( \alpha/\beta \), and \( \alpha \)-tubulin and the phosphorylation of GSK-3\( \alpha/\beta \) were determined by Western blotting. C, HepG2 cells were transfected with mature SREBP1a, either wild-type or the indicated mutants. Thirty-six hours after transfection, the cells were treated in the absence or presence of LY294002 (5 \( \mu \)M) for 2 h. The expression of SREBP1, GSK-3\( \alpha/\beta \), and \( \alpha \)-tubulin and the phosphorylation of GSK-3\( \alpha/\beta \) were determined by Western blotting. D, HepG2 cells were treated with insulin for 2 h in the absence or presence of LY294002 (5 \( \mu \)M) and rapamycin (50 nM). The inhibitors were added 30 min before the addition of insulin. The expression of SREBP1, GSK-3\( \alpha/\beta \), S6K, and \( \alpha \)-tubulin and the phosphorylation GSK-3\( \alpha/\beta \) and S6K (ptT379) were determined by Western blotting.

binding-dependent phosphorylation of Ser-434 was attenuated in the presence of lithium (Fig. 5D), suggesting that GSK-3 contributes to the phosphorylation of Ser-434 in response to DNA binding. This effect was specific for lithium and was not seen with other kinase inhibitors tested (supplemental Fig. S6). Our earlier results suggested that Ser-434 regulates the GSK-3-dependent phosphorylation of Thr-426 and Ser-430. To test if this was also true in response to DNA binding, the kinase assays were repeated with recombinant SREBP1, either wild-type or the S434A mutant, in the absence or presence of DNA. As seen in Fig. 5E, the phosphorylation of both Thr-426 and Ser-430 in response to DNA binding was almost completely lost in the S434A mutant, confirming that Ser-434 plays an important role in the phosphorylation of both these residues.

To further define the role of Ser-434 in GSK-3-dependent phosphorylation of SREBP1, we used synthetic peptides corresponding to the region surrounding Ser-434 in SREBP1 in peptide pulldown assays with extracts from HeLa cells. GSK-3\( \alpha/\beta \)and \( \beta \) were recruited to the non-phosphorylated peptide, and the interaction was enhanced when the peptide was phosphorylated on Ser-434, whereas the interaction was unaffected in the S434A mutant peptide (Fig. 5F). Interestingly, phosphorylation of Ser-430 greatly enhanced the recruitment of GSK-3 to the peptide (Fig. 5G), indicating that the interaction between GSK-3 and SREBP1 is enhanced in response to phosphorylation of both Ser-430 and Ser-434. Taken together, our results demonstrate that Ser-434 in the mature form of SREBP1a, as well as the corresponding residue in SREBP1c, is phosphorylated by GSK-3, both in vitro and in vivo. The phosphorylation of Ser-434 regulates the stability of SREBP1 by promoting its degradation. In addition, the phosphorylation of Ser-434 is important for the subsequent phosphorylation of Thr-426 and Ser-430 in the phosphodegron in SREBP1.

Phosphorylation of Ser-434 regulates the Fbw7-dependent Degradation of SREBP1—The interaction between Fbw7 and SREBP1 is dependent on the phosphorylation of Thr-426 and Ser-430 (9). Our data suggest that the phosphorylation of Thr-426 and Ser-430 is dependent on Ser-434, suggesting that the interaction between SREBP1 and Fbw7 could be regulated by Ser-434. To address this issue, we performed coimmunoprecipitation assays between Fbw7 and SREBP1a, either wild-type or the S434A mutant. Wild-type SREBP1 interacted with Fbw7, whereas the S434A mutant failed to interact (Fig. 6A). These results suggest that the phosphorylation of Ser-434 could control the interaction between SREBP1 and Fbw7 by regulating the phosphorylation of Thr-426 and Ser-430 in the phosphodegron. To further define the role of Ser-434 phosphorylation in the interaction between SREBP1 and Fbw7, we used synthetic peptides corresponding to the phosphodegron in SREBP1 in peptide pulldown assays with Fbw7\( \alpha \). As demonstrated earlier (9), the binding of Fbw7\( \alpha \) to the peptide was strongly dependent on the phosphorylation of Thr-426 and Ser-430 (supplemental Fig. S7). Interestingly, phosphorylation of Ser-434 did not affect the binding of Fbw7 to the peptide, supporting the notion that phosphorylation of Ser-434 regulates the interaction between SREBP1 and Fbw7 in vivo by controlling the phosphorylation of Thr-426 and Ser-430.

Thus, our results indicate that SREBP molecules that are not phosphorylated on Ser-434, such as the S434A mutant, would be poor substrates for Fbw7-mediated degradation in vivo. To test this hypothesis, HEK293T cells were transfected with mature SREBP1a, either wild-type or the S434A mutant, in the absence or presence of increasing amounts of Fbw7\( \alpha \). In support of our hypothesis, the S434A protein was less sensitive to Fbw7\( \alpha \)-mediated degradation compared with the wild-type protein (Fig. 6B). In addition, transfected wild-type mature SREBP1c was stabilized in response to shRNA-mediated inactivation of Fbw7 in HepG2 cells, whereas the S410A mutant was stable under normal conditions and insensitive to inactivation of Fbw7 (Fig. 6C). These results are in agreement with our observation that the Ser-434 mutant accumulates when expressed in cells and suggest that phosphorylation of Ser-434 could be important for Fbw7-dependent degradation of mature SREBP1. We have earlier demonstrated that mature SREBP1 accumulates in Fbw7-negative cells and that the protein is highly phosphorylated on both Thr-426 and Ser-430, suggest-
Phosphorylation-dependent Degradation of SREBP1

Phosphorylation of Ser-434 regulates the phosphodegron in SREBP1. A, HEK293T cells were transfected with FLAG-SREBP1a, either wild-type or the indicated mutants, and WCE were immunoprecipitated with anti-FLAG antibodies. The levels of SREBP1a and the phosphorylation of SREBP1a on Thr-426, Ser-430, and Ser-434 were determined by Western blotting. B, recombinant His<sub>6</sub>-SREBP1a, either wild-type or the S434A mutant, was incubated with GSK-3β. The levels and phosphorylation on pT426 and pS430 of SREBP1a were determined by Western blotting. C, recombinant His<sub>6</sub>-SREBP1a, either wild-type or the S430A mutant, was incubated with GSK-3β. The levels and phosphorylation on pT426 of SREBP1a were determined by Western blotting. D, recombinant His<sub>6</sub>-SREBP1a was incubated with HeLa nuclear extract in the absence or presence of NaCl or LiCl (20 mM). The levels and phosphorylation on pT426, pS430, and pS434 of SREBP1a were determined by Western blotting. E, recombinant His<sub>6</sub>-SREBP1a, either wild-type or the S434A mutant, was incubated with HeLa nuclear extract in the absence or presence of a plasmid DNA template containing the proximal region of the HMG-CoA synthase promoter and in the presence of NaCl or LiCl (20 mM). The levels and phosphorylation on pT426, pS430, and pS434 of SREBP1a were determined by Western blotting. F, WCE from HeLa cells were used in peptide pulldown assays, using three separate peptides corresponding to residues 422–442 of human SREBP1a, either unphosphorylated (Ref), the same peptide phosphorylated on Ser-434 (pS434), or the same peptide containing the S434A mutation. The bound proteins were subjected to SDS-PAGE and Western blotting using 20% of input as control. G, WCE from HeLa cells were used in peptide pulldown assays, using two separate peptides corresponding to residues 422–442 of human SREBP1a, either unphosphorylated (Ref) or the same peptide phosphorylated on Ser-430 (pS430). The bound proteins were subjected to SDS-PAGE and Western blotting using 20% of input as control.

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1

Phosphorylation-dependent Degradation of SREBP1
Phosphorylation-dependent Degradation of SREBP1

As illustrated in Fig. 7C, insulin enhanced the levels of SREBP1c in the presence of control shRNA. Inactivation of Fbw7 enhanced the levels of transfected mature SREBP1c, and no further increase was observed in response to insulin treatment. To determine if phosphorylation of Ser-434/410 could regulate the accumulation of mature SREBP1c in response to insulin, HepG2 cells were transfected with mature SREBP1c, either wild-type or the S410A mutant. The levels of wild-type SREBP1c were enhanced in response to insulin treatment, whereas the levels of the S410A mutant were high in nonstimulated cells and failed to respond to insulin (Fig. 7D), suggesting that the phosphorylation of Ser-434/410 could be a target for insulin-dependent regulation of mature SREBP1.

Our results suggest that Fbw7 could affect insulin-dependent regulation of mature SREBP1. To determine if Fbw7 could regulate SREBP-dependent transcription, HepG2 cells were transfected with three different SREBP-dependent promoter-reporter genes and either control or Fbw7 shRNA. As expected, shRNA-mediated inactivation of Fbw7 enhanced the expression of all three promoter-reporter genes in the absence of insulin treatment (Fig. 7E). Importantly, inactivation of Fbw7 attenuated the insulin-dependent activation of all three promoter-reporters, suggesting that Fbw7 could regulate SREBP1 function in response to insulin signaling. This possibility was supported by our observation that the insulin-dependent phosphorylation of Ser-434 in SREBP1 promotes the phosphorylation of two residues in its C-terminal domain, Thr-426 and Ser-430 in its phosphodegron. Consequently, the phosphorylation of Ser-434 regulates the interaction between the phosphodegron in SREBP1 and the Fbw7 ubiquitin ligase, thereby controlling the degradation of mature SREBP1. The phosphorylation of Ser-434 is also enhanced in response to DNA binding, and mutation of Ser-434 blocks the phosphorylation of Thr-426 and Ser-430 during this process, suggesting that the phosphorylation of Ser-434 could control the degradation of active SREBP1 molecules. In addition, our results suggest that the ubiquitin ligase Fbw7 could play an important role in insulin-dependent regulation of nuclear SREBP1. Thus, the degradation of mature SREBP1 is controlled by a phosphorylation cascade, and the phosphorylation of Ser-434 could function as a molecular switch to control these processes.

DISCUSSION

The mature form of the transcription factor SREBP1 is degraded by the ubiquitin-proteasome system following phosphorylation of two residues in its C-terminal domain, Thr-426 and Ser-430 in SREBP1a (Thr-402 and Ser-406 in SREBP1c) (9, 12). Phosphorylation of these residues creates a docking site for the ubiquitin ligase SCF<sup>Fbw7</sup>, which catalyzes the polyubiquitination of mature SREBP1. Both residues are phosphorylated by GSK-3β in vitro, and inactivation of GSK-3 in cells attenuates the phosphorylation of both residues, suggesting that GSK-3 contributes to the phosphorylation of the SREBP1 phosphodegron in vivo. Here we identify an additional phosphorylated residue in the C-terminal domain of mature SREBP1, Ser-434 (S410 in SREBP1c). Phosphorylation of Ser-434 in SREBP1a promotes the phosphorylation of its phosphodegron, thereby enhancing the recruitment of Fbw7 and Fbw7-dependent degradation of SREBP1. Thus, the degradation of mature SREBP1 is controlled by cross-talk between multiple phosphorylated Ser and Thr residues in its C-terminal domain.
In vitro kinase assays with recombinant SREBP1a and GSK-3β followed by phosphopeptide mapping indicated that Ser-434 could be phosphorylated by GSK-3β in vitro. This notion was confirmed using phospho-S434-specific antibodies. These antibodies also recognized endogenous SREBP1, and the phosphorylation of Ser-434 in mature SREBP1. However, our results do not exclude the involvement of other kinases, and a more detailed analysis of the kinases targeting Ser-434 in vivo is warranted.

We propose that inhibition of the phosphorylation-dependent degradation of the active transcription factor by SCF/Fbw7 could be an early target of insulin signaling, thereby contributing to a feed-forward mechanism resulting in enhanced expression of SREBP target genes. Taken together, the data in this report indicate that GSK-3 is involved in the phosphorylation of Ser-434 in mature SREBP1. However, our results do not exclude the involvement of other kinases, and a more detailed analysis of the kinases targeting Ser-434 in vivo is warranted.

Treatment of cells with lithium not only attenuated the phosphorylation of Ser-434, but also enhanced the amount of nuclear SREBP1. In addition, SREBP1 molecules phosphorylation-dependent Degradation of SREBP1
Phosphorylation-dependent Degradation of SREBP1

A, a phosphorylation cascade controls the Fbw7-dependent degradation of mature SREBP1. GSK-3 phosphorylates Ser-434 in the C terminus of SREBP1, thereby creating a binding site for GSK-3, which results in enhanced GSK-3-dependent phosphorylation of Ser-430. Phosphorylation of Ser-430 generates a high affinity binding site for GSK-3, which enhances the GSK-3-dependent phosphorylation of Thr-426. Phosphorylation of Thr-426 and Ser-430 in turn creates a binding site for the ubiquitin ligase SCFFbw7, resulting in the polyubiquitination and degradation of mature SREBP1. The phosphorylation of all three residues is attenuated by insulin signaling, thereby contributing to the insulin-dependent accumulation of mature SREBP1. Thus, the degradation of mature SREBP1 is controlled by cross-talk between multiple phosphorylated residues in its C-terminal domain, and the phosphorylation of Ser-434 could function as a molecular switch to control these processes. B, Fbw7 could regulate SREBP1 on different levels. Fbw7 negatively regulates mature SREBP1 by targeting it for proteasome-mediated degradation following phosphorylation of its phosphodegron. In addition, Fbw7 also targets mTor for degradation (39). mTor has been reported to promote the activation of premature SREBP1 (40). Thus, Fbw7 could be a negative regulator of both the activation of SREBP, as well as the function of the active transcription factor.

Phosphorylation-dependent Degradation of SREBP1 also has a Ser residue at the position corresponding to Ser-434 in SREBP1a (Ser-440 in SREBP2). However, Ser-440 in SREBP2 is not followed by a Pro residue, and mutation of Ser-440 results in destabilization of the protein (data not shown), indicating that the mechanisms regulating the phosphorylation and degradation of SREBP1 and -2 could differ.

As indicated above, mutation of Ser-434 attenuated the phosphorylation of Thr-426 and Ser-430 in transfected cells. Interestingly, the phosphorylation of Thr-426 and Ser-430 by recombinant GSK-3β in vitro was significantly reduced in the S434A mutant, suggesting that Ser-434 directly enhances GSK-3-dependent phosphorylation of Thr-426 and Ser-430. At the same time, we could demonstrate that the GSK-3-dependent phosphorylation of Thr-426 was dependent on the phosphorylation of Ser-430. Using peptide pulldown assays, we found that phosphorylation of Ser-434 enhanced the interaction between GSK-3 and a peptide containing Thr-426 and Ser-430, and that the phosphorylation of Ser-430 further enhanced this interaction. Phosphorylation of many GSK-3 substrates requires a priming phosphorylation four residues C-terminal to the GSK-3 phosphorylation site (33, 35, 36). We propose that the
phosphorylation of Ser-434 could be such a priming phosphorylation event for the subsequent phosphorylation of Ser-430. Furthermore, once Ser-430 is phosphorylated, it serves as a priming site for GSK-3-dependent phosphorylation of Thr-426. In turn, phosphorylation of Thr-426 and Ser-430 creates a high affinity docking site for Fbw7, resulting in rapid degradation of the protein (Fig. 8A). The synergistic phosphorylation of Ser-434, Ser-430, and Thr-426 sensitizes SREBP1 toward changes in GSK-3 activity. In addition, this mechanism of phosphorylation increases the probability that individual SREBP1 molecules are phosphorylated on both Ser-430 and Thr-426, thereby making these molecules more susceptible to degradation. GSK-3 substrates that require prior phosphorylation are usually targeted by a separate kinase, a so-called priming kinase. GSK-3 substrates that require prior phosphorylation of Ser-434, Ser-430, and Thr-426 sensitizes SREBP1 toward changes in GSK-3 activity. In addition, this mechanism of phosphorylation increases the probability that individual SREBP1 molecules are phosphorylated on both Ser-430 and Thr-426, thereby making these molecules more susceptible to degradation. GSK-3 substrates that require prior phosphorylation are usually targeted by a separate kinase, a so-called priming kinase. GSK-3 substrates that require prior phosphorylation of Ser-434, Ser-430, and Thr-426 sensitizes SREBP1 toward changes in GSK-3 activity. In addition, this mechanism of phosphorylation increases the probability that individual SREBP1 molecules are phosphorylated on both Ser-430 and Thr-426, thereby making these molecules more susceptible to degradation.

Taken together, our results demonstrate that Fbw7 regulates the stability of active SREBP1. However, it is possible that Fbw7 regulates SREBP1 in other ways as well. It was recently demonstrated that Fbw7 targets mTor for degradation (39). Interestingly, it has been suggested that mTor is a positive regulator of the acute response to activation of premature SREBP1 (40). Thus, Fbw7 could regulate both the mature and premature forms of SREBP1 (Fig. 8B). However, inhibition of mTor by rapamycin failed to affect the insulin-dependent regulation of SREBP1 in HepG2 cells. We only treated cells with insulin for short times in our experiments, suggesting that mTor does not play an important role in the acute response to activation of the insulin signaling pathway. However, the role of mTor, as well as the Fbw7-dependent degradation of mTor, in the regulation of SREBP1 will be an important subject for future studies.

Acknowledgments—We thank Ulla Engström for peptide synthesis and antibody purification and Vasily Lukiyanchuk for technical assistance.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11041–11048
2. Osborne, T. F. (2000) J. Biol. Chem. 275, 32379–32382
3. Bengoechea-Alonso, M. T., and Ericsson, J. (2007) Curr. Opin. Cell Biol. 19, 215–222
4. Espenshade, P. J. (2006) J. Cell Sci. 119, 973–976
5. Rawson, R. B. (2003) Nat. Rev. Mol. Cell Biol. 4, 631–640
6. Goldstein, J. L., Debose-Boyd, R. A., and Brown, M. S. (2006) Cell 124, 35–46
7. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) J. Clin. Investig. 109, 1125–1131
8. Kotzka, J., Lehr, S., Roth, G., Avci, H., Knebel, B., and Muller-Wieland, D. (2004) J. Biol. Chem. 279, 22404–22411
9. Sundqvist, A., Bengoechea-Alonso, M. T., Ye, X., Lukiyanchuk, V., Jin, J., Harper, J. W., and Ericsson, J. (2005) Cell Metab. 1, 379–391
10. Bengoechea-Alonso, M. T., Punta, T., and Ericsson, J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 11681–11686
11. Bengoechea-Alonso, M. T., and Ericsson, J. (2006) Cell Cycle 5, 1708–1718
12. Punta, T., Bengoechea-Alonso, M. T., and Ericsson, J. (2006) J. Biol. Chem. 281, 25278–25286
13. Giandomenico, V., Simonsson, M., Gronroos, E., and Ericsson, J. (2003) Mol. Cell. Biol. 23, 2587–2599
14. Hirano, Y., Murata, S., Tanaka, K., Shimizu, M., and Sato, R. (2003) J. Biol. Chem. 278, 16809–16819
15. Sundqvist, A., and Ericsson, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 13833–13838
16. Hirano, Y., Yoshida, M., Shimizu, M., and Sato, R. (2001) J. Biol. Chem. 276, 36431–36437
17. Muratani, M., and Tansey, W. P. (2003) Nat. Rev. Mol. Cell Biol. 4, 192–201
18. Lipford, J. R., and Deshaies, R. J. (2003) Nat. Cell Biol. 5, 845–850
19. Welcker, M., and Churman, B. E. (2007) Nat. Rev. Cancer 7, 83–93
20. Koepp, D. M., Schafer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Science 294, 173–177
21. Strohmaier, H., Spruck, C. H., Kaiser, P., Won, K. A., Sangfelt, O., and Reed, S. I. (2001) Nature 413, 316–322
22. Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., and Nakayama, K. I. (2004) EMBO J. 23, 2115–2125
23. Welcker, M., Orián, A., Jin, J., Grim, J. E., Harper, J. W., Eisenman, R. N., and Churman, B. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9085–9090
24. Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M., and Lendahl, U. (2001) J. Biol. Chem. 276, 35847–35853
25. Wu, G., Lyapina, S., Das, I., Li, J., Gurney, M., Pauley, A., Chui, I., Deshaies, R. J., and Kitajewski, J. (2001) Mol. Cell. Biol. 21, 7403–7415
26. Tetzlaff, M. T., Yu, W., Li, M., Zhang, P., Finegold, M., Mahon, K., Harper, J. W., Schwartz, R. J., and Elledge, S. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3338–3345
27. Tsunematsu, R., Nakayama, K., Oiko, Y., Nishiyama, M., Ishida, N., Hatakeyama, S., Bessho, Y., Kageyama, R., Suda, T., and Nakayama, K. I. (2004) J. Biol. Chem. 279, 9417–9423
28. Wei, W., Jin, J., Schlissel, S., Harper, J. W., and Kaelin, W. G., Jr. (2005) Cancer Cell 8, 25–33
29. Olson, B. L., Hock, M. B., Ekholm-Reed, S., Wohlschlegel, J. A., Dev, K. K., Kralli, A., and Reed, S. I. (2008) Genes Dev. 22, 252–264
30. Wu, R. C., Feng, Q., Lonard, D. M., and O’Malley, B. W. (2007) Cell 129, 1125–1140
31. Rajagopalan, H., Jallepalli, P. V., Rago, C., Velculescu, E. V., Kinzler, K. W., Vogelstein, B., and Lengauer, C. (2004) Nature 428, 77–81
32. Ericsson, J., Jackson, S. M., and Edwards, P. A. (1996) J. Biol. Chem. 271, 24359–24364
33. Cohen, P., and Frame, S. (2001) Nat. Rev. Mol. Cell Biol. 2, 769–776
34. Ferre, P., and Foufelle, F. (2007) Horm. Res. (Basel) 68, 72–82
35. Doble, B. W., and Woodgett, J. R. (2003) J. Cell Sci. 116, 1175–1186
36. Jope, R. S., and Johnson, G. V. (2004) Trends Biochem. Sci. 29, 95–102
37. Frame, S., and Cohen, P. (2001) Biochem. J. 359, 1–16
38. Forde, J. E., and Dale, T. C. (2007) Cell. Mol. Life Sci. 64, 1930–1944
39. Mao, J.-H., Kim, I.-J., Wu, D., Climent, J., Kang, H. C., DelRosario, R., and Balmain, A. (2008) Science 321, 1499–1502
40. Porstmann, T., Santos, C. R., Griffiths, B., Cully, M., Wu, M., Levers, S., Griffiths, I. R., Chung, Y. L., and Schulze, A. (2008) Cell Metab. 8, 224–236

Phosphorylation-dependent Degradation of SREBP1