Proteolytic Activation of Sterol Regulatory Element-binding Protein Induced by Cellular Stress through Depletion of Insig-1*

Joon No Lee and Jin Ye†‡
From the Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9046

Insig-1 and Insig-2 are closely related proteins of the endoplasmic reticulum (ER) that block proteolytic activation of sterol regulatory element-binding proteins (SREBPs), membrane-bound transcription factors that activate synthesis of cholesterol and fatty acids in animal cells. When cellular cholesterol levels are high, Insig proteins bind to SREBP cleavage-activating protein, retaining it in the ER and preventing it from escorting SREBPs to the site of proteolytic activation in the Golgi complex. Here we report that hypotonic stress reverses the sterol-mediated inhibition of SREBP proteolytic activation by reducing the level of Insig-1 but not Insig-2. The reduction of Insig-1, a protein with a rapid turnover rate, results from a general inhibition of protein synthesis mediated by hypotonic stress. Insig-2 is not affected by hypotonic stress because of its slower turnover rate. Inhibition of protein synthesis by hypotonic shock has not been reported previously. Thapsigargin, an activator of the ER stress response, also inhibits protein synthesis and activates proteolysis of SREBP. Such activation also correlates with the disappearance of Insig-1. The current study demonstrates that animal cells, in response to either hypotonic shock or ER stress, can bypass the cholesterol inhibition of SREBP processing, an effect that is attributable to the rapid turnover of Insig-1.

SREBPs1 are a family of membrane-bound transcription factors that control the synthesis of cholesterol and fatty acids in animal cells (1). After synthesis in ER membranes, SREBPs form a complex with SCAP. When cells are depleted of sterols, SCAP escorts SREBPs from the ER to the Golgi. Within the Golgi two resident proteases, Site-1 protease (S1P) and Site-2 protease (S2P), sequentially cleave the SREBPs through regulated intramembrane proteolysis, release the NH2-terminal fragment of SREBP from the membrane, and allow it to translocate to the nucleus and activate transcription of target genes (2). When sterols accumulate within cells, either by de novo synthesis or uptake of cholesterol through the LDL receptor, SCAP undergoes a conformational change, which causes the SCAP-SREBP complex to be retained in the ER and thus abrogates SREBP-dependent transcription.

Insig-1 and Insig-2 were recently identified as membrane proteins that reside in the ER and play a central role in the regulation of SREBP proteolytic processing (3, 4). When cellular cholesterol levels are elevated, SCAP binds to Insigs, an action that blocks the movement of the SREBP/SCAP complex from the ER to the Golgi, thus blocking proteolytic cleavage and transcriptional activation of SREBPs. In cultured cells, the net result of Insig action is to decrease lipid synthesis whenever sterols accumulate to high levels within membranes.

Human Insig-1 is composed of 277 amino acids (3), whereas human Insig-2 contains 225 amino acids (4). Both are deeply embedded in ER membranes through the presence of six membrane-spanning segments (5). The proteins exhibit an amino acid identity of 59% with the differences confined mostly to the hydrophilic NH2- and COOH-terminal regions. Insig-1 and Insig-2 are functionally similar in that both cause the ER retention of the SCAP-SREBP complex (3, 4), although their transcription is regulated differently: Insig-1 expression is positively controlled by nuclear SREBPs, while Insig-2 expression is negatively regulated by insulin (6).

In the current study, we report that another difference between Insig-1 and Insig-2 is the turnover rate of Insig-1 is much quicker than that of Insig-2. We show that two cellular perturbations that inhibit general protein synthesis, incubation of cells in hypotonic medium or thapsigargin-induced ER stress (7), deplete Insig-1 because of its intrinsically rapid rate of degradation. The depletion of Insig-1 allows SREBP to be proteolytically activated even in the presence of sterols. The results indicate that mammalian cells can rapidly bypass cholesterol-mediated feedback inhibition by decreasing the synthesis of Insig-1.

EXPERIMENTAL PROCEDURES

Materials—We obtained sorbitol, thapsigargin, RPMI 1640 amino acid solution, and RPMI 1640 vitamin solution from Sigma; sucrose from Invitrogen; all sterols from Steraloids, Inc.; herpessirusid herpesvirus inclusion complex (11) were prepared as described.

Plasmid Constructs—The following plasmids were described in the indicated references: pCMV-Myc-S2P, encoding Myc epitope-tagged human S2P driven by the CMV enhancer/promoter (13); pCMV-Myr-Myc, encoding Myc epitope-tagged human S2P driven by CMV enhancer/promoter (13); and pCMV-Insig-1-Myc and pCMV-Insig-2-Myc, encoding full-length versions of human Insig-1 and Insig-2, respectively.
forms of SREBP-2, respectively. and immunoblotted with anti-SREBP-2 antibody. Aliquots of membranes and nuclear extracts were subjected to SDS-PAGE and immunoblotted with anti-SREBP-2 antibody. Filters were exposed for 10 s. P and N denote precursor and nuclear forms of SREBP-2, respectively.

and human Insig-2, respectively, followed by six tandem copies of a c-Myc epitope tag under control of the CMV enhancer/promoter (3, 4).

Cell Culture—Cells were maintained in monolayer culture at 37 °C in 8–9% CO2. CHO-7 cells are a clone of CHO-K1 cells selected for growth in lipoprotein-deficient serum (15). SRD-12B cells (15) and SRD-13A cells (SCAPΔC) (17) are mutant cells derived from CHO-7 cells. M19 cells (2PΔ) are mutant cells derived from CHO-K1 cells (13, 18). CHO/pInsig-1-Myc cells are a clone of CHO-7 cells stably transfected with pCMV-Insig-1-Myc (3). CHO/pInsig-2-Myc cells were generated by stable transfection of CHO-7 cells with pCMV-Insig-2-Myc (4).

Stock cultures of CHO-7 cells were maintained in medium A (1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 5% (v/v) newborn calf lipoprotein-deficient serum. M19, SRD-12B, and SRD-13A cells were maintained in medium B (medium A supplemented with 5% (v/v) fetal calf serum, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μM sodium oleate). CHO/pInsig-1-Myc and CHO/pInsig-2-Myc cells were maintained in medium B containing 500 μg/ml G418.

Medium C contains 20 mM NaCl, 5 mM KCl, 1 mM CaCl2, 0.7 mM MgSO4, 0.34 mM NaH2PO4, 4.17 mM NaHCO3, 0.44 mM KH2PO4, 5.55 mM glucose, 1X RPMI 1640 amino acid solution, 1X RPMI vitamin solution, 1X sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. At room temperature, the pH of medium C is 7.4–7.5.

In all experiments, the designation of “normal medium” refers to medium A, “hypotonic medium” refers to medium A supplemented with 50 mM sucrose, and “isotonic medium” refers to medium C supplemented with 50 mM sucrose.

Transient Transfection of Cells and Immunoblot Analysis—CHO-7, M19, SRD-12B, and SRD-13A cells were transiently transfected with FuGene 6 reagent (Roche Applied Science) according to the manufacturer’s protocol. Conditions of incubation after transfection are described in the figure legends. After incubation, duplicate dishes of cells were pooled, harvested, and fractionated into membranes and nuclear extracts as described previously (8). Thirty μl of membranes and nuclear extracts were subjected to SDS-PAGE and immunoblot analysis.

Antibodies were used at the following concentrations: IgG-7D4, a mouse monoclonal antibody against Myc tag, 1 μg/ml; and horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgGs, 0.2 μg/ml. Bound antibodies were visualized by chemiluminescence using the SuperSignal substrate system (Pierce) according to the manufacturer’s instructions. Filters were exposed to Kodak X-OMat Blue XB-1 films at room temperature for the indicated time.

Real-time PCR—The protocol was identical to that described by Liang et al. (19). Triplicate samples of first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with hamster actin as an invariant control. Relative amounts of mRNAs were calculated using the comparative Ct method.

Pulse-Chase Analysis in CHO-7 Cells—CHO-7 cells stably transfected with Myc-tagged Insig-1 or Insig-2 were incubated in medium A supplemented with 5% FCS as described above. Twenty h after incubation, cells were washed with phosphate-buffered saline and switched to medium D (methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After 1 h at 37 °C, the cells were pulse-labeled with 150 μCi/ml 35S protein labeling mix (PerkinElmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 h, the medium was removed and the cells were washed with phosphate-buffered saline and chased for various times in the indicated medium supplemented with 0.5 mM unlabeled methionine and 1 mM unlabeled cysteine. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline, lysed in 0.7 ml of lysis buffer, and analyzed by SDS-PAGE and immunoblot analysis.

The protocol was identical to that described by Liang et al. (19). Triplicate samples of first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with hamster actin as an invariant control. Relative amounts of mRNAs were calculated using the comparative Ct method.

Pulse-Chase Analysis in CHO-7 Cells—CHO-7 cells stably transfected with Myc-tagged Insig-1 or Insig-2 were incubated in medium A supplemented with 5% FCS as described above. Twenty h after incubation, cells were washed with phosphate-buffered saline and switched to medium D (methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After 1 h at 37 °C, the cells were pulse-labeled with 150 μCi/ml 35S protein labeling mix (PerkinElmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 h, the medium was removed and the cells were washed with phosphate-buffered saline and chased for various times in the indicated medium supplemented with 0.5 mM unlabeled methionine and 1 mM unlabeled cysteine. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline, lysed in 0.7 ml of lysis buffer, and analyzed by SDS-PAGE and immunoblot analysis.

The protocol was identical to that described by Liang et al. (19). Triplicate samples of first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with hamster actin as an invariant control. Relative amounts of mRNAs were calculated using the comparative Ct method.

Pulse-Chase Analysis in CHO-7 Cells—CHO-7 cells stably transfected with Myc-tagged Insig-1 or Insig-2 were incubated in medium A supplemented with 5% FCS as described above. Twenty h after incubation, cells were washed with phosphate-buffered saline and switched to medium D (methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After 1 h at 37 °C, the cells were pulse-labeled with 150 μCi/ml 35S protein labeling mix (PerkinElmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 h, the medium was removed and the cells were washed with phosphate-buffered saline and chased for various times in the indicated medium supplemented with 0.5 mM unlabeled methionine and 1 mM unlabeled cysteine. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline, lysed in 0.7 ml of lysis buffer, and analyzed by SDS-PAGE and immunoblot analysis.

The protocol was identical to that described by Liang et al. (19). Triplicate samples of first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with hamster actin as an invariant control. Relative amounts of mRNAs were calculated using the comparative Ct method.

Pulse-Chase Analysis in CHO-7 Cells—CHO-7 cells stably transfected with Myc-tagged Insig-1 or Insig-2 were incubated in medium A supplemented with 5% FCS as described above. Twenty h after incubation, cells were washed with phosphate-buffered saline and switched to medium D (methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After 1 h at 37 °C, the cells were pulse-labeled with 150 μCi/ml 35S protein labeling mix (PerkinElmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 h, the medium was removed and the cells were washed with phosphate-buffered saline and chased for various times in the indicated medium supplemented with 0.5 mM unlabeled methionine and 1 mM unlabeled cysteine. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline, lysed in 0.7 ml of lysis buffer, and analyzed by SDS-PAGE and immunoblot analysis.

The protocol was identical to that described by Liang et al. (19). Triplicate samples of first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with hamster actin as an invariant control. Relative amounts of mRNAs were calculated using the comparative Ct method.

Pulse-Chase Analysis in CHO-7 Cells—CHO-7 cells stably transfected with Myc-tagged Insig-1 or Insig-2 were incubated in medium A supplemented with 5% FCS as described above. Twenty h after incubation, cells were washed with phosphate-buffered saline and switched to medium D (methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After 1 h at 37 °C, the cells were pulse-labeled with 150 μCi/ml 35S protein labeling mix (PerkinElmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 h, the medium was removed and the cells were washed with phosphate-buffered saline and chased for various times in the indicated medium supplemented with 0.5 mM unlabeled methionine and 1 mM unlabeled cysteine. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline, lysed in 0.7 ml of lysis buffer, and analyzed by SDS-PAGE and immunoblot analysis.

The protocol was identical to that described by Liang et al. (19). Triplicate samples of first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with hamster actin as an invariant control. Relative amounts of mRNAs were calculated using the comparative Ct method.

Pulse-Chase Analysis in CHO-7 Cells—CHO-7 cells stably transfected with Myc-tagged Insig-1 or Insig-2 were incubated in medium A supplemented with 5% FCS as described above. Twenty h after incubation, cells were washed with phosphate-buffered saline and switched to medium D (methionine/cysteine-free Dulbecco’s modified Eagle’s medium containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). After 1 h at 37 °C, the cells were pulse-labeled with 150 μCi/ml 35S protein labeling mix (PerkinElmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 h, the medium was removed and the cells were washed with phosphate-buffered saline and chased for various times in the indicated medium supplemented with 0.5 mM unlabeled methionine and 1 mM unlabeled cysteine. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline, lysed in 0.7 ml of lysis buffer, and analyzed by SDS-PAGE and immunoblot analysis.
resuspended in 100 μl of 1× SDS loading buffer, and boiled for 5 min. After centrifugation at 16,000 × g for 3 min at room temperature, the supernatant was transferred to a fresh tube, and aliquots of the immunoprecipitates were subjected to SDS-PAGE. Radiolabeled proteins were transferred to Hybond C-Extra nitrocellulose filters. The filters were dried, exposed to an imaging plate at room temperature for 1 h, and scanned in a Fuji X Bas 1000 phosphorimaging device.

RESULTS

Fig. 1 shows the content of SREBP-2 in membrane and nuclear extracts of CHO-7 cells that were incubated for 2 h in media with varying osmolarities, all containing 5% FCS. When cells were incubated in normal culture medium containing 5% FCS, we observed the full-length precursor form of SREBP-2 in cell membranes but no cleaved mature form in nuclear extracts (Fig. 1, lane 1). This finding indicates that the LDL in FCS provides enough cholesterol to suppress SREBP cleavage. To test whether this suppression can be reversed by hypotonic stress, we created a medium with lower osmolarity by reducing NaCl from 140 to 20 mM (medium C). When cells were incubated in medium C containing 20 mM NaCl plus 5% FCS, we observed the cleaved nuclear form of SREBP-2 in nuclear extracts with a dramatic decrease of the precursor form in the membranes (Fig. 1, lane 2), indicating that SREBP cleavage occurred even though cells were exposed to the same amount of LDL cholesterol. This effect was abolished when we raised the osmolarity by adding more than 50 mM NaCl (Fig. 1, lanes 4–7), 200 mM sorbitol (Fig. 1, lanes 13 and 14), or 100 mM sucrose (Fig. 1, lanes 19–21). Inasmuch as sorbitol and sucrose were effective in restoring inhibition of SREBP-2 cleavage, these data indicate that the stimulation of SREBP-2 cleavage seen in medium C is because of hypotonic stress, not NaCl deprivation. Similar results were obtained when these membranes and nuclear extracts were immunoblotted with an antibody against SREBP-1 (data not shown). We also found that hypotonic stress stimulates SREBP cleavage in three other types of mammalian cells: human embryo kidney cells (HEK-293 cells), SV-40 transformed human fibroblasts (SV589 cells), and human hepatoma cells (Huh-7 cells) (data not shown). The hypotonic shock that we used in our studies is not lethal to cells, as cells continued to grow normally after they were switched back to normal medium.

In all studies described below, we define hypotonic medium as medium C containing 20 mM NaCl plus 50 mM sucrose, and isotonic medium as medium C containing 20 mM NaCl plus 200 mM sucrose. As a further control, we studied normal medium, which contains 140 mM NaCl and no sucrose.

The data of Fig. 1 demonstrate that hypotonic stress can
reverse the suppression of SREBP proteolytic processing mediated by the LDL present in 5% FCS. We then tested whether the effect of hypotonic stress is specific for LDL-derived cholesterol. For this purpose, CHO-7 cells were incubated in 5% lipoprotein-deficient serum supplemented with 25-hydroxycholesterol delivered in ethanol, complexes of cholesterol-methyl-β-cyclodextrin, or LDL for 16 h prior to and during exposure to the normal, hypotonic, or isotonic medium. As shown in Fig. 2A, all three sterol delivery vehicles effectively suppressed SREBP-2 cleavage in normal (Fig. 2A, lanes 1, 4, and 7) and isotonic medium (Fig. 2A, lanes 3, 6, and 9) but not in hypotonic medium (Fig. 2A, lanes 2, 5, and 8). These data indicate that hypotonic stress reverses sterol-mediated inhibition of SREBP proteolytic processing regardless of whether the sterol is 25-hydroxycholesterol, cholesterol delivered in β-methyl-β-cyclodextrin, or cholesterol delivered in LDL. Inasmuch as these three sources of sterol reach the ER by different mechanisms, these results indicate that hypotonic stress interferes with regulation at a step distal to ER delivery of sterol. This conclusion is supported by the observation that LDL stimulates cholesterol esterification normally in hypotonically shocked cells (data not shown), a finding that implies that LDL cholesterol reaches the ER membrane where esterification takes place.

To determine whether nuclear SREBP that is released through hypotonic stress is transcriptionally active, we measured mRNA levels of SREBP target genes using real-time PCR. Actin was used as the invariant control. The concentrations of hydroxymethylglutaryl-CoA synthase and hydroxymethylglutaryl-CoA reductase mRNAs, two well characterized SREBP targets, were enhanced 4- and 2-fold, respectively, in hypotonic but not in isotonic medium. The mRNA of Insig-2, a gene that is not subject to SREBP regulation, did not change upon hypotonic stress. The situation for Insig-1, another SREBP downstream gene, was more complicated. Its mRNA transcript was increased in both hypotonic and isotonic media. We do not understand the factor(s) that lead to increased Insig-1 mRNA in the high sucrose isotonic medium. Similar results were obtained in two other experiments performed on different days.

We next performed a series of experiments in wild type and mutant CHO cells to determine whether hypotonic stress-induced cleavage of SREBP requires the same proteins that are required for SREBP processing upon sterol deprivation. For this purpose, we transfected the cells with a plasmid (pTK-HSV-SREBP2) encoding human SREBP-2 tagged with two copies of an NH2-terminal epitope derived from an HSV glycoprotein. To avoid artifacts from gross overexpression, the epitope-tagged SREBP-2 is driven by the weak HSV thymidine kinase promoter, which gives a low level of expression that is detectable by immunoblotting with HSV antibody. In wild type CHO-7 cells, the proteolytic cleavage of transfected SREBP-2 was stimulated by hypotonic stress (Fig. 3A). In mutant CHO cells deficient in SCAP (Fig. 3B), no nuclear cleaved form of SREBP-2 was observed when cells were incubated with hypotonic medium (Fig. 3B, lane 3), but co-transfection with SCAP restored the cleavage (Fig. 3B, lane 6). We also observed that the precursor form of SREBP-2 disappeared when these cells were incubated in hypotonic medium, regardless of whether SCAP was present or absent (Fig. 3B, lanes 3 and 6). The significance of this result will be discussed below. In mutant CHO cells deficient in S1P (Fig. 3C),
Hypotonic and ER Stress Stimulates SREBP Cleavage

Hypotonic stress did not induce SREBP-2 cleavage (Fig. 3C, lane 2). Co-transfection with S1P restored the cleavage of SREBP-2 under hypotonic stress conditions (Fig. 3C, lane 5). In mutant CHO cells deficient in S2P (Fig. 3D), there was no nuclear cleaved form of SREBP-2 with or without hypotonic stress. However, when we transfected a cDNA encoding S2P, we restored the ability of hypotonic stress to induce nuclear SREBP (Fig. 3D, lane 6). Fig. 3D also shows the membrane-bound intermediate form of SREBP that represents the product of site-1 cleavage; it is only seen in cells lacking S2P. This intermediate was present in the cells incubated in normal medium containing FCS, primarily because these cells do not take up cholesterol from LDL in the FCS, due to a lack of LDL receptors, which is attributable to the lack of nuclear SREBPs. The data in Fig. 3, A–D, indicate that hypotonic stress-induced proteolytic processing of SREBP requires SCAP, S1P, and S2P, the same proteins that are required for SREBP processing upon sterol deprivation.

The above data suggest that the SCAP-SREBP complex is able to move from ER to Golgi despite the presence of ER sterols in cells subjected to hypotonic stress. To determine whether the failure to block SCAP-SREBP movement is attributable to a loss of Insig-1 or Insig-2, we measured the levels of these proteins (as well as SCAP) by immunoblotting. Inasmuch as we lack an antibody sensitive enough to detect endogenous Insigs in CHO cells, we used CHO-7 cells stably transfected with Myc-tagged Insig-1 and Insig-2 (CHO/pInsig-1-Myc) (Fig. 4A) or CHO/pInsig-2-Myc (Fig. 4B). As shown in Fig. 4A, incubation of CHO/pInsig-1-Myc cells in hypotonic medium stimulated SREBP cleavage as early as 1 h, with the peak induction occurring at 2 h (Fig. 4A, lanes 5–7). A similar increase was not observed in normal or isotonic medium (Fig. 4A, lanes 2–4 and 8–10). The abundance of SCAP and Insig-2 protein did not change throughout the incubation time regardless of which medium was used. Insig-1 protein showed two bands that represent translatable products initiated with different methionines (3). Both bands showed a 50% reduction at 1 h and an 80% reduction at 2 h after switching to hypotonic medium (Fig. 4A, lanes 5 and 6). Such reduction did not occur when cells were incubated in normal and isotonic media. These data raised the possibility that a selective reduction of Insig-1 protein during hypotonic stress may be responsible for stimulation of SREBP proteolytic processing.

The decline in Insig-1 protein after hypotonic stress occurred in the face of an increase in Insig-1 mRNA (Fig. 2B). We thus reasoned that hypotonic stress must decrease Insig-1 protein synthesis or increase its degradation. To distinguish these alternatives, we performed a pulse-chase experiment. CHO/pInsig-1-Myc cells were pulse-labeled for 1 h with 35S-labeled methionine plus cysteine, after which they were washed and switched to normal, hypotonic, or isotonic medium containing an excess of unlabeled methionine and cysteine. Radiolabeled Myc-tagged Insig-1 was monitored by immunoprecipitation with anti-Myc antibodies followed by SDS-PAGE and visualization in a phosphorimaging device. As shown in Fig. 5A in all three incubation media, the radiolabeled Insig-1 disappeared rapidly, with a half-life between 1 and 2 h and an almost complete disappearance after 4 h. We also performed a pulse-chase experiment with CHO/pInsig-2-Myc cells. As shown in Fig. 5B, there was no significant reduction in radiolabeled Insig-2 during the 4-h chase in all three types of media. These data indicate that the rate of degradation of Insig-1 is rapid and unaffected by hypotonic stress. This finding implies that the reduction of Insig-1 in hypotonic stress must be due to a reduction in synthesis.

To test the hypothesis that the synthesis of Insig-1 protein is reduced during hypotonic stress, we pulse-labeled the cells during incubation in media with varying osmolarities. CHO/pInsig-1-Myc cells were pre-incubated in normal, hypotonic, and isotonic media for 1 h before they were pulse-labeled for 1 h with 35S-labeled methionine plus cysteine. An aliquot of cell lysate was used for quantification of the total rate of protein synthesis, and the rest was immunoprecipitated with anti-Myc antibody. The synthesis of total protein and Insig-1 was monitored by immunoprecipitation, followed by SDS-PAGE and visualization in a phosphorimaging device. As shown in Fig. 6, the synthesis of total protein was not affected by hypotonic stress, whereas the synthesis of Insig-1 was reduced by 50%. These data indicate that the reduced synthesis of Insig-1 is responsible for the decrease in Insig-1 protein during hypotonic stress.

**FIG. 4.** Insig-1 protein diminishes upon hypotonic stress. CHO-7 cells stably transfect with Myc-tagged Insig-1 (A) or Insig-2 (B) were set up at 7 × 10^6 cells/60-mm dish on day 0. They were changed to medium A containing 5% FCS on day 1. On day 2, cells were switched to normal (N), hypotonic (H), and isotonic (I) media containing 5% FCS for the indicated time. Cells were then harvested and fractionated. Aliquots of membranes and nuclear extracts were subjected to SDS-PAGE and immunoblotted with anti-SREBP-2, anti-SCAP, and anti-Myc antibodies. Filters were exposed 1–3 s. P and N denote precursor and nuclear forms of SREBP-2, respectively.
respectively. Fig. 5C shows that synthesis of Insig-1 protein was demonstrable in normal and isotonic media but was barely detectable in hypotonic medium (Fig. 5C, upper panel). The rate of total protein synthesis was also reduced more than 50% in hypotonic medium, judging by both the decrease in $^{35}$S-labeled protein in the supernatant from the immunoprecipitation (Fig. 5C, lower panel) and the quantification of protein synthesis by scintillation counting (Fig. 5D). These data indicate that synthesis of Insig-1 as well as total protein synthesis is reduced under hypotonic stress.

If depletion of Insig-1 protein is responsible for hypotonic stress-induced SREBP cleavage, then overexpressing Insig-1 should inhibit such cleavage. To test the hypothesis, we transfected pTK-HSV-SREBP2 into CHO-7 cells with or without co-transfection of Myc-tagged Insig-1 driven by the CMV promoter (Fig. 6A). When pTK-HSV-SREBP2 was transfected
alone, hypotonic stress stimulated SREBP-2 cleavage (Fig. 6A, lanes 2–4). When Insig-1 was co-transfected, the nuclear form of SREBP-2 was barely detectable after hypotonic stress (Fig. 6A, lane 6). Hypotonic stress did not reduce the level of transfected Insig-1 protein in these cells, probably because of the pronounced overexpression.

As observed in Fig. 6A, we noticed that the membrane-bound precursor form of SREBP-2 was reduced by hypotonic treatment regardless of whether Insig-1 was co-transfected. This disappearance most likely reflects the normal degradation of the SREBP precursor that is independent of its proteolytic cleavage. In normal medium, this degradation process is presumably balanced by continued protein synthesis, but in hypotonic medium the SREBP-2 precursor declines because of the overall decrease in protein synthesis. This scenario may also explain the disappearance of the SREBP-2 precursor observed in Fig. 3B, lanes 3 and 6.

As a control for the specificity of the Insig-1 deficiency, we co-transfected Insig-2 together with pTK-HSV-SREBP2. As shown in Fig. 6B, overexpressing Insig-2 to the same level as that of Insig-1 (Fig. 6A) did not significantly reduce hypotonic stress-induced SREBP-2 cleavage. We therefore conclude that under conditions of hypotonic stress, Insig-2 cannot substitute for Insig-1 in blocking SREBP cleavage.

The above data suggest that hypotonic stress inhibits protein synthesis, thereby reducing the level of the rapidly turning over Insig-1 protein, and this reduction in turn stimulates SREBP proteolytic processing. If this scenario is true, other conditions of cellular stress that inhibit protein synthesis should also activate SREBP cleavage. Protein synthesis is inhibited in one well recognized condition, namely ER stress caused by the accumulation of misfolded proteins in the lumen of the ER. To test that hypothesis, we examined the cleavage of SREBP after treatment with thapsigargin, an agent that disturbs protein folding in the ER through calcium depletion. This stress activates PERK, a protein kinase that phosphorylates and inactivates the translation initiation factor, eIF2α (7), thereby inhibiting protein synthesis. CHO/pInsig-1-Myc cells were incubated in normal medium containing 5% FCS in the presence of various amounts of thapsigargin. As shown in Fig. 7A, thapsigargin stimulated SREBP-2 cleavage in a dose-dependent manner. Insig-1 protein also decreased upon thapsigargin treatment. The dose of thapsigargin required for activation of SREBP-2 cleavage correlated well with that required for reduction of the Insig-1 protein.

We also pulse-labeled the cells after thapsigargin treatment to measure the synthesis of total protein and Insig-1 by monitoring the supernatant and pellet, respectively, of immunoprecipitation mediated by anti-Myc antibody. As shown in Fig. 7B, thapsigargin reduced the synthesis of Insig-1 protein as well as total protein synthesis. These data indicate that in addition to hypotonic shock, other forms of cellular stress that inhibit protein synthesis can also activate SREBP proteolytic processing by depleting the Insig-1 protein.
DISCUSSION

In the current study we found that two cellular perturbations, incubation of cells in hypotonic medium and thapsigargin-induced ER stress, each inhibit general protein synthesis. It is well documented that ER stress inhibits protein synthesis by activating PERK, a protein kinase that phosphorylates and inhibits the translation initiation factor, eIF2α (7, 21). In contrast, the finding that hypotonic stress inhibits general protein synthesis is an observation that to our knowledge has not been previously reported. The inhibition of protein synthesis leads to the depletion of Insig-1, a protein with a very rapid rate of turnover. The disappearance of Insig-1 protein in turn allows SREBP proteolytic activation, which is supported by the observation that overexpression of Insig-1 inhibits hypotonic stress-induced SREBP cleavage.

Unlike Insig-1, Insig-2 is a stable protein with a much slower turnover rate. It is not rapidly depleted upon cellular stress that inhibits protein synthesis. However, endogenous Insig-2 does not appear to substitute for the function of Insig-1, probably because of its low level of expression (22). It is also possible that Insig-2 is inactivated upon hypotonic shock, as overexpression of Insig-2 was not able to suppress the cleavage of SREBP-2 in cells incubated in hypotonic medium (Fig. 6B).

The physiologic role of nuclear SREBP generated during hypotonic shock or ER stress remains unclear. In several experiments, we did not observe an increase in either fatty acid or cholesterol synthesis upon hypotonic shock and ER stress (data not shown). It is puzzling why a transcription factor like SREBP should be activated under stress conditions when protein synthesis is inhibited. However, a precedent exists. ATF6, another membrane-bound transcription factor that undergoes regulated intramembrane proteolysis, is also cleaved and sent to the nucleus upon ER stress (23, 24). As with SREBPs, the target mRNAs of nuclear ATF6 are increased by ER stress, but they may not be functional because of the general inhibition of protein synthesis by PERK activation (21).

One prediction of the current studies is that chemical inhibitors of protein synthesis, such as cycloheximide, should block Insig-1 synthesis and thereby stimulate the cleavage of SREBPs. Unfortunately, we were unable to test this hypothesis because cycloheximide treatment blocked SREBP cleavage even in cholesterol-depleted cells in normal medium. Apparently, cycloheximide blocks synthesis of one or more rapidly turning over proteins, which are essential for ER-to-Golgi transport. The inhibition of protein synthesis that occurs with ER stress or hypotonic shock may be more selective, sparing the putative cycloheximide-inhibitable proteins so that ER-to-Golgi transport can take place.

The current study demonstrates that cells can change their feedback response to cholesterol by regulating the protein level of Insig-1. In the case of hypotonic shock and ER stress, depletion of Insig-1 is achieved by inhibition of protein synthesis. The rapid turnover rate of Insig-1 is crucial for its depletion under these conditions. Judging from our observations in stably transfected cells overexpressing Insig-1, we calculate that the half-life of Insig-1 is less than 2 h, which is similar to that of hydroxymethylglutaryl-CoA reductase exposed to sterols and mevalonate (25). The mechanism and regulation of the rapid turnover of Insig-1 is a new area for future study.
Acknowledgments—We thank Drs. Michael S. Brown and Joseph L. Goldstein for their constant support, helpful comments, and critical review of the manuscript; Lorena Avila for excellent technical assistance; Lisa Beatty, Angela Carroll, and Jill Fairless for assistance in tissue culture; and Jeff Cormier and Erin Friedman for real-time PCR analysis.

REFERENCES

Hypotonic and ER Stress Stimulates SREBP Cleavage 45265