Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake

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Summary

This paper describes a convergent mechanism for the feedback control of cholesterol synthesis and uptake mediated by SREBPs, membrane bound transcription factors. Endoplasmic reticulum (ER) bound SREBPs form complexes with Scap, a polytopic ER protein. In sterol-overloaded cells, Scap/SREBP binds to Insig-1, which retains the complex in the ER. Upon sterol deprivation, the Scap/SREBP complex dissociates from Insig-1, which is then ubiquitinated on lysines 156 and 158 and degraded in proteasomes. Scap/SREBP moves to the Golgi, where SREBP is processed to liberate a nuclear fragment that activates genes for cholesterol synthesis and uptake and the gene for Insig-1. Ubiquitination is not necessary for release of Scap/SREBP from Insig-1, but it establishes a requirement for synthesis of new Insig-1 for feedback inhibition. When the new Insig-1 and cholesterol converge on Scap, Scap/SREBP binds to Insig-1, preventing ubiquitination. The Insig-1/Scap/SREBP complex accumulates in the ER, ready for liberation when the cell is again sterol deprived.

Introduction

Insigs are polytopic membrane proteins of the endoplasmic reticulum (ER) that regulate lipid synthesis by retaining sterol regulatory element binding proteins (SREBPs) in the ER and preventing their proteolytic activation in the Golgi apparatus (Yang et al., 2002; Yabe et al., 2002). SREBPs are transcription factors that are synthesized as integral ER membrane proteins. Immediately after synthesis, they form tight complexes with Scap, a membrane-embedded escort protein (Brown and Goldstein, 1999). When membrane cholesterol levels are low, the Scap/SREBP complex is incorporated into COPII-coated vesicles that bud from the ER and carry the SREBPs to the Golgi, where the concerted actions of two proteases release the transcription-factor portion of the SREBP into the cytosol, from which it enters the nucleus (Nothurff et al., 2000; Espenshade et al., 2002; Sun et al., 2005). There, the SREBPs activate transcription by binding to sterol regulatory elements in the enhancers of more than 20 genes that produce enzymes required for synthesis of cholesterol and its uptake by low-density lipoprotein (LDL) receptors (Horton et al., 2003). When cholesterol accumulates in ER membranes, the sterol binds to Scap (Radhakrishnan et al., 2004), altering its conformation and causing the Scap/SREBP complex to bind to Insigs (Brown et al., 2002; Adams et al., 2004).

When the Scap/SREBP complex binds to Insigs, it can no longer be incorporated into COPII-coated vesicles (Sun et al., 2005). The ER-retained SREBPs cannot be processed, and transcription of the target genes declines. This feedback-suppression mechanism allows cholesterol to control its own synthesis and uptake, and it ensures a steady level of cholesterol in cell membranes (Goldstein et al., 2002). Binding of Scap/SREBP complexes to Insigs can also be triggered by certain oxygenated derivatives of cholesterol, including 25-hydroxycholesterol (25-HC) (Adams et al., 2004).

Because the formation of Scap/SREBP/Insig complexes is stoichiometric rather than catalytic, the sterol regulatory system depends on the relative amounts of Scap and Insig in ER membranes. Under artificial conditions, relative overexpression of Scap saturates endogenous Insigs, and the excess Scap transports SREBPs to the Golgi, even in the presence of sterols (Yang et al., 2002). On the other hand, overexpression of Insigs causes the Scap/SREBP complex to be retained in the ER even at low levels of cellular cholesterol, thereby inappropriately limiting cholesterol synthesis (Yang et al., 2002; Yabe et al., 2002; Engelking et al., 2005). To prevent these imbalances, elaborate transcriptional and posttranscriptional mechanisms control the concentration of Insigs.

Cells express two Insig genes that encode proteins that are 59% identical and appear to serve redundant functions (Yabe et al., 2002; Engelking et al., 2005). The two Insig proteins differ in their mechanisms of control. Insig-1 is an SREBP target gene (Yang et al., 2002; Horton et al., 2002). Its mRNA is expressed at high levels when nuclear SREBP levels are high as a result of sterol deprivation. Because of this dependence on SREBPs, Insig-1 mRNA levels decline drastically when cells are overloaded with sterols. In cultured cells, the amount of Insig-2 is lower than that of Insig-1, even when the cells have been overloaded with sterols. Importantly, Insig-2 is not regulated by SREBPs, and hence it persists when cells are overloaded with sterols and SREBPs are excluded from the nucleus (Sever et al., 2004; Lee et al., 2005).

Recently, a new level of potential control was uncovered with the observation that Insig-1 is degraded much more rapidly than Insig-2 (Lee and Ye, 2004). This rapid turnover of Insig-1 occurred when protein synthesis was blocked in cultured cells by exposing them to hypotonic stress, causing a rapid disappearance of Insig-1. In the current studies, we have sought to determine whether the rapid degradation of Insig-1 is regulated. The results indicate that Insig-1 is rapidly ubiquitinated and...
degraded only when it is free from Scap and that sterol-induced binding of Insig-1 to Scap prevents Insig-1 ubiquitination and degradation. These results lead to a new model for the convergent inhibition of SREBP processing and cholesterol supply in animal cells.

**Results**

**Insig-1 protein is stabilized by sterols**

Figure 1 shows a pulse-chase experiment demonstrating that Insig-1 is rapidly degraded in sterol-depleted cells and that this degradation is blocked by sterols in the form of 25-HC. Wild-type CHO cells were depleted of sterols by prior incubation in medium containing lipoprotein-deficient serum (LPDS), the sterol synthesis inhibitor compactin (an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMG CoA reductase]), and a low concentration of mevalonate to prevent depletion of nonsterol endproducts (Goldstein and Brown, 1990). The cells were then pulse labeled with $^{35}$S)methionine plus $^{35}$S)cysteine and chased in isotope-free medium. Insig-1 was isolated by immunoprecipitation, subjected to SDS-PAGE, and the $^{35}$S-labeled protein was visualized in a phosphorimager. Insig-1 was degraded only when it is free from Scap and that sterol-induced binding of Insig-1 to Scap prevents Insig-1 ubiquitination and degradation. These results lead to a new model for the convergent inhibition of SREBP processing and cholesterol supply in animal cells.

**Sterol stabilization of Insig-1 requires Scap**

Figure 3A shows that the ability of 25-HC to stabilize Insig-1 depends on the presence of Scap. For this purpose, we used SRD-14/pTK-Insig1-Myc cells to study Insig-1 degradation without radioisotopes, we used SDS-PAGE and immunoblotting to follow the disappearance of total Insig-1 after protein synthesis was blocked by cycloheximide (CHX) (Figure 2). In the absence of sterols, endogenous Insig-1 disappeared from wild-type CHO cells within 30 min after CHX addition (Figure 2A, lanes 1–3). This rapid degradation was blocked by 25-HC (Figure 2A, lanes 6–10). Similar results were obtained when we used CHX to follow the degradation of epitope-tagged Insig-1 that was produced by transfection from a constitutive nonregulated promoter (Figure 2B).

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Sterol-regulated degradation of Insig-1

Figure 3. Slowing of Insig-1 degradation requires sterol-dependent interaction with Scap and is blocked by proteasome inhibitors

On day 0, Scap-deficient SRD-13A cells were set up at 3.5 x 10⁵ cells per 60 mm dish in medium C.

A) On day 2, cells were transfected in 3 ml of medium A containing 5% FCS with 1 μg pTK-Insig1-Myc (lanes 1–12) with (lanes 7–12) or without (lanes 1–6) 1 μg pTK-Scap as indicated. The total DNA in each dish was adjusted to 3 μg/dish by addition of pcDNA3 mock vector. Six hours after transfection, cells were refed with sterol-depleting medium B. After 16 hr (see schematic), cells were switched to fresh medium B containing 50 μM CHX in the absence (−) or presence (+) of 1 μg/ml of 25-HC. At the indicated time after CHX treatment, cells were harvested and fractionated, and aliquots of whole-cell lysates were subjected to SDS-PAGE and immunoblot analysis with 5 μg/ml monoclonal anti-Myc IgG (against Insig-1) and 5 μg/ml polyclonal IgG-R139 (against Scap). Filters were exposed to film for 30 s (lanes 1–6) and 5 s (lanes 7–12) for Insig-1 and for 1 min for Scap (lanes 1–12).

B) On day 2, cells were transfected in 3 ml of medium A containing 5% FCS with 0.2 μg pTK-Insig1-Myc and 1 μg of either wild-type (wt) pTK-Scap or a mutant version (Y298C) as indicated. The total DNA was adjusted to 3 μg/dish by addition of pcDNA3 mock vector. Six hours after transfection, cells were refed with sterol-depleting medium B. After 16 hr (see schematic), cells were switched to fresh medium B containing 10 μM MG-132 (lanes 7–12) in the absence (−) or presence (+) of 1 μg/ml 25-HC. After incubation for 5 hr, cells were harvested and fractionated, and aliquots of whole-cell lysates were subjected to SDS-PAGE and immunoblot analysis with 5 μg/ml of monoclonal anti-Myc IgG (against Insig-1) and 5 μg/ml polyclonal IgG-R139 (against Scap). Filters were exposed to film for 5 s (Insig-1) or 1 min (Scap).

was retarded even in the absence of 25-HC (lanes 7–9), likely because the cells contained a low level of residual sterols. Degradation was further blocked when 25-HC was added (lanes 10–12).

To confirm that Scap binding mediates the stabilization of Insig-1, we transfected the SRD-13A cells with a plasmid encoding Scap(Y298C), a mutant Scap that does not bind to Insigs (Adams et al., 2004). In SRD-13A cells producing Insig-1-Myc alone, Insig-1-Myc levels were low in either the absence or presence of 25-HC (Figure 3B, lanes 1 and 2). Coexpression of wild-type Scap stabilized Insig, even in the absence of exogenous sterols (lane 3), and stabilization was increased by 25-HC (lane 4). Coexpression of Scap(Y298C) did not stabilize Insig-1, even in the presence of 25-HC (lanes 5 and 6). Immunoblotting with an antibody against Scap confirmed that equal amounts of wild-type and Y298C were expressed (Figure 3B, bottom panel, lanes 3–6). When we incubated the cells with MG-132, an inhibitor of the proteasome, Insig-1 became visible even in the absence of Scap (lanes 7) and in cells expressing Scap(Y298C) (lane 11), and there was no further effect of 25-HC (lanes 8, 10, and 12). These data indicate that Insig-1 is rapidly degraded by a proteasomal process and that full stabilization requires the presence of 25-HC and a form of Scap that is capable of binding to Insig.

Ubiquitination precedes Insig-1 degradation

Proteasomal degradation of a protein requires prior ubiquitination (Hershko and Ciechanover, 1998). To demonstrate ubiquitination of Insig-1, we transfected Scap-deficient SRD-13A cells with a plasmid encoding Insig-1 and a plasmid encoding ubiquitin that contains an HA epitope tag. The cells were incubated in sterol-depleting medium, after which some of the dishes received 25-HC. For 2 hr prior to harvest, the cells were incubated with MG-132 to block the degradation of ubiquitinated Insig-1. Insig-1 was immunoprecipitated from detergent-solubilized lysates, and the precipitates were subjected to SDS-PAGE and blotted with an antibody to the HA tag on ubiquitin. In control cells not expressing T7-tagged Insig-1, no ubiquitin was seen in the Insig-1 immunoprecipitates (Figure 4A, lane 1). In cells expressing the tagged Insig-1, the anti-ubiquitin blot of the Insig-1 immunoprecipitate exhibited the typical smear seen with polyubiquitinated proteins (lane 2). The most rapidly migrating bands consisted of a doublet that migrated at the same position as authentic Insig-1. Since they were visualized with the anti-HA antibody, these bands likely represented Insig-1 that contained a minimal number of ubiquitins. In the absence of Scap, the addition of 25-HC did not affect Insig-1 ubiquitination (lane 3). Coexpression of Scap did not affect Insig-1 ubiquitination in the absence of sterols (lane 4), but it permitted 25-HC to block this ubiquitination (lane 5). Although ubiquitinated Insig-1 was clearly seen when blotted with the antibody against the HA tag in ubiquitin, we did not see a higher-molecular-weight smear (data not shown) when we blotted with an antibody against the T7 tag on Insig-1 (IB: Insig-1 in Figure 4A). Although Figure 4A shows only a portion of the Insig-1 immunoblot, we also did not observe a smear when the whole autoradiogram was examined. This is the usual finding in studies of protein ubiquitination in intact cells, and it is believed to reflect the activity of deubiquitinating enzymes that remove the ubiquitin when cell extracts are made. The amount of residual ubiquitinated Insig-1 is sufficient for visualization when blotted for ubiquitin but not when blotted for Insig-1.

We reasoned that ubiquitination of Insig-1 likely occurs on lysine residues oriented toward the cytoplasm. Insig-1 is predicted to contain four such residues at positions 33, 156, 158, and 273 (Feramisco et al., 2004). In preliminary mutagenesis experiments, we determined that the lysines at positions 33 and 273 could be changed to arginines without affecting ubiquitination (data not shown). However, the closely spaced lysines at

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residues 156 and 158 were crucial. Figure 4B shows that wild-type Insig-1 was ubiquitinated as determined by immunoprecipitation of Insig-1 followed by blotting with anti-HA ubiquitin (lane 4). No ubiquitination was demonstrated when we expressed a plasmid encoding a mutant of Insig-1 with arginines substituted for lysines at positions 156 and 158 (lane 5). The bottom autoradiogram of Figure 4B shows that equal amounts of wild-type and mutant Insigs were precipitated with the antibody against the T7 epitope on the Insigs. Mutant Insig-1 with only a single lysine mutation (K156R or K158R) was degraded as rapidly as wild-type Insig-1, suggesting that ubiquitination on either lysine is sufficient for proteasomal degradation (data not shown).

Ubiquitination of Insig-1 is not required for its dissociation from Scap
If ubiquitination is required for rapid degradation of Insig-1, then the K156R/K158R mutant should not be degraded, and it should not require Scap and 25-HC for stabilization. To test this hypothesis, we used SRD-14 cells, a line of mutant CHO cells that does not express Insig-1 but does express Scap (Sever et al., 2004). We transfected the SRD-14 cells with plasmids encoding wild-type Insig-1 or the K156R/K158R mutant under control of the thymidine kinase (TK) promoter. Stable lines expressing wild-type and mutant Insig-1 were prepared. In the absence of sterols, immunoblot analysis with 1:1000 dilution of polyclonal anti-HA IgG (against ubiquitin), 1:15,000 dilution of polyclonal anti-T7 IgG (against Insig-1), and 5 μg/ml polyclonal IgG-R139 (against Scap). Filtres were exposed to film for 5 s.

Figure 4. Lysine 156 and lysine 158 are required for sterol-mediated ubiquitination and degradation of Insig-1 in hamster cells
A) On day 0, Scap-deficient SRD-13A cells were set up at 3.5 \times 10^5 cells per 60 mm dish in medium C. On day 2, cells were transfected in 3 ml of medium A containing 5% FCS with 0.2 μg of pEF1a-HA-ubiquitin, 0.2 μg pCMV-Insig1-T7, and 1 μg of pCMV-Scap as indicated. The total DNA in each lane was adjusted to 3 μg/dish by addition of pcDNA3 mock vector. Six hours after transfection, cells were refed with sterol-depleting medium B. After 16 hr, cells were switched to fresh medium B in the absence (–) or presence (+) of 1 μg/ml of 25-HC. After incubation for 3 hr, each dish received 10 μM MG-132. Following incubation at 37°C for 2 hr, cells were harvested and subjected to immunoprecipitation with monoclonal anti-T7 IgG-coupled agarose beads. Aliquots of cell lysates and immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis with 1:1000 dilution of polyclonal anti-HA IgG (against ubiquitin), 1:15,000 dilution of polyclonal anti-T7 IgG (against Insig-1), and 5 μg/ml polyclonal IgG-R139 (against Scap). Filtres were exposed to film for 5 s.

B) On day 0, SRD-13A cells were set up as in (A). On day 2, cells were transfected in 3 ml of medium A containing 5% FCS with 0.2 μg of pEF1a-HA-ubiquitin and/or 0.2 μg of wt pCMV-Insig1-T7 or a mutant (m) version (K156R/K158R) as indicated. The total DNA in each lane was adjusted to 3 μg/dish by addition of pcDNA3 mock vector. After 16 hr, each dish received 10 μM MG-132. Following incubation at 37°C for 2 hr, cells were harvested and subjected to immunoprecipitation, SDS-PAGE, and immunoblot analysis as above. Filtres were exposed to film for 2–20 s.

C) On day 0, SRD-14/pTK-Insig1-Myc cells (lanes 1–10) and SRD-14/pTK-Insig1(K156R/K158R)-Myc cells (lanes 11–20) were set up at 5 \times 10^5 cells per 100 mm dish in medium A with 5% LPDS. On day 2, cells were refed with sterol-depleting medium B. After 16 hr, cells were switched to fresh medium B containing 50 μM CHX in the absence (–) or presence (+) of 1 μg/ml of 25-HC. At the indicated time after CHX treatment, cells were harvested and fractionated, and aliquots of whole-cell lysates were subjected to 10% SDS-PAGE and immunoblot analysis with 2 μg/ml of monoclonal anti-Myc IgG against Insig-1. Filtres were exposed to film for 30 s (lanes 1–10) and 2 s (lanes 11–20).
wild-type Insig-1 or the K156R/K158R mutant. We tested the response of these cells to two types of sterol depletion: (1) gradual depletion by incubating the cells in serum from which the cholesterol-carrying lipoproteins had been removed (LPDS) (Figure 5A) and (2) rapid depletion by incubating the cells in hydroxypropyl-β-cyclodextrin (HPCD) (Figure 5B). Prior to the start of both experiments, the cells were incubated with 25-HC to form the stable Insig-1/Scap/SREBP complex. The amount of Insig-1 and the amount of Scap that coimmunoprecipitated with Insig-1 were measured. We also measured the amount of nuclear SREBP-2. In the gradual sterol-depletion experiment (Figure 5A), in cells expressing wild-type Insig-1, the amount of Insig-1 began to decline as early as 4 hr after initiating sterol depletion. At this time point, there was a modest decline in the amount of Scap that coimmunoprecipitated with Insig-1, and a trace of nuclear SREBP-2 appeared (lane 2). By 8 hr, Insig-1 had declined further, we no longer visualized any Scap immunoprecipitated with Insig-1, and nuclear SREBP-2 was clearly detectable (lane 3). A delay in these processes was seen in the cells expressing the K156R/K158R mutant. At 8 hr, the amount of the mutant Insig-1 remained high, abundant Scap continued to coimmunoprecipitate with the Insig-1, and nuclear SREBP-2 was still not visible (lane 8). By 24 hr, the mutant Insig-1 had declined, the amount of Scap in the immunoprecipitate fell, and nuclear SREBP-2 was clearly visible (lane 10). These data suggest that, under conditions of mild sterol depletion, the failure to ubiquitinate Insig-1 causes a delay in release of the Scap/SREBP-2 complex, but it does not abolish it.

**Figure 5.** Time course of SREBP cleavage in Insig-1-deficient hamster cells stably transfected with wild-type or mutant Insig-1 after gradual or acute depletion of sterols

On day 0, SRD-14/pTK-Insig1-Myc cells (lanes 1–5) and SRD-14/pTK-Insig1(K156R/K158R)-Myc cells (lanes 6–10) were set up at 7 × 10^5 cells per 60 mm dish in medium A with 5% LPDS. On day 1, cells were refed with medium A containing 5% LPDS and 0.3 μg/ml of 25-HC.

A) After incubation for 12 hr with 25-HC, the cells were washed and then switched to sterol-free medium B for the indicated time.

B) After cells were incubated for 16 hr with 25-HC, the cells were treated with 1% (w/v) hydroxypropyl-β-cyclodextrin (HPCD) in sterol-free medium B for 15 min, then washed and switched to sterol-free medium B for the indicated time. In (A) and (B), the switch in medium was staggered such that all the cells could be harvested at the same time and divided into two groups. One group of cells was fractionated, and aliquots of whole-cell lysates were subjected to 8 or 10% SDS-PAGE and immunoblot analysis with 1 μg/ml of monoclonal anti-Myc IgG (against Insig-1) and 5 μg/ml of monoclonal IgG-7D4 (against SREBP-2). P and N denote precursor and nuclear forms of SREBP-2, respectively. The second group of cells was immunoprecipitated (IP) with 40 μg/ml polyclonal anti-Myc against Insig-1 as described in Experimental Procedures. Immunoprecipitated pellets (representing 0.25 dish of cells) and supernatants (representing 0.05 dish of cells) were subjected to 8 or 10% SDS-PAGE and immunoblotted (IB) with 5 μg/ml monoclonal IgG-9D5 (against Scap) and 1 μg/ml anti-Myc monoclonal IgG (against Insig-1). Filters were exposed to film for 2–60 s.

**Table 1.** Time course of SREBP cleavage in Insig-1-deficient hamster cells stably transfected with wild-type or mutant Insig-1 after gradual or acute depletion of sterols

<table>
<thead>
<tr>
<th>Time after Sterol Depletion (hours)</th>
<th>Sterol-depleting medium (hours)</th>
<th>Whole Cell Lysate</th>
<th>Insig-1</th>
<th>SREBP-2</th>
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**Table 2.** Time course of SREBP cleavage in Insig-1-deficient hamster cells stably transfected with wild-type or mutant Insig-1 after gradual or acute depletion of sterols

<table>
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<tr>
<th>Time after Sterol Depletion (hours)</th>
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<th>Whole Cell Lysate</th>
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The data of Figure 5B indicate that, upon severe sterol depletion to suppress SREBP cleavage (lane 8). Nevertheless, within 1 hr, Scap no longer immunoprecipitated with Insig-1, and nuclear SREBP-2 was already being generated (lane 8).

**Cholesterol and new Insig-1 converge on Scap to suppress SREBP cleavage**

The data of Figure 5B indicate that, upon severe sterol depletion, the Scap/SREBP complex can dissociate from Insig-1 even when Insig-1 is not ubiquitinated or degraded. Thus, ubiquitination of Insig-1 is not required in order for Insig-1 to release Scap. What, then, is the role of ubiquitination and degradation? We hypothesized that this degradation is necessary in order to establish a condition in which SREBP processing can only be terminated when SREBPs have entered the nucleus, activated the Insig-1 gene, and restored the level of Insig-1 protein.

To test this hypothesis, we performed sterol-depletion experiments in wild-type cells that express Insig-1 from its natural promoter (Figures 6A–6D). In Figures 6A and 6B, wild-type CHO-7 cells were preincubated with 25-HC and then treated for 15 min with cycloheximide to deplete sterols, followed by incubation in medium with LPDS. At zero time, Insig-1 was present, and there was no detectable nuclear SREBP-2 (Figure 6A). Insig-1 protein was observed despite a relatively low level of Insig-1 mRNA as measured by quantitative real-time PCR (see bar graph in Figure 6B), presumably because Insig-1 was protected from degradation by formation of the sterol-dependent Scap/Insig-1 complex. Within 0.75 hr of sterol depletion, most of the Insig-1 had been degraded, nuclear SREBP-2 became visible, and the amount of Insig-1 mRNA had risen by 3-fold owing to transcriptional stimulation by nuclear SREBPs. The amount of Insig-1 protein began to increase as the level of Insig-1 mRNA rose. At 1.5 hr, FCS was added to the culture medium. In the Golgi, the newly synthesized Insig-1 will be de

The data of Figures 6A and 6B, together with previous data in this paper, indicate that, upon acute sterol deprivation, the increase in nuclear SREBPs stimulates the uptake and synthesis of cholesterol as well as the synthesis of new Insig-1 molecules. The convergence of cholesterol and Insig-1 traps the Scap/SREBP complex in the ER, leading to a decline in nuclear SREBPs and Insig-1 mRNA, but a persistence of Insig-1 protein, owing to the stability of its complex with Scap. To confirm that cholesterol uptake is required for this convergent effect, we repeated the sterol-depletion experiment, but this time we incubated the cells in lipoprotein-deficient serum (LPDS) instead of FCS. In contrast to FCS, LPDS is devoid of LDL, and it cannot supply cholesterol to the cells. The results are shown in Figures 6C and 6D. When cells were depleted of sterols and then placed in FCS, Insig-1 protein initially declined and nuclear SREBP-2 increased (Figure 6C). Thereafter, the reciprocal changes occurred, i.e., Insig-1 protein rose and nuclear SREBP-2 declined. These results duplicate the findings of Figure 6A. When the sterol-depleted cells were incubated in LPDS, the initial response was the same, i.e., Insig-1 fell and nuclear SREBP-2 increased (Figure 6D). However, in the absence of LDL cholesterol, the subsequent rise in Insig-1 was delayed and there was no fall in nuclear SREBP-2. This result indicates that the cells require the convergence of cholesterol and Insig-1 in order to block SREBP processing. Endogenous cholesterol synthesis is not sufficient to satisfy the cholesterol requirement over the short term of this experiment, and therefore, LDL-derived cholesterol is essential.

Figures 6E and 6F show the result opposite to that in Figures 6A–6D—namely, the response of sterol-depleted CHO cells to the addition of 25-HC. In this experiment, the cells were preincubated in LPDS to deplete sterols, and then 25-HC was added. At zero time, the sterol-deprived cells contained a relatively small amount of Insig-1 protein, no Scap was communoprecipitated with Insig-1, and the content of nuclear SREBP-2 was high (Figure 6E). The amount of Insig-1 mRNA was also high, presumably owing to the high level of nuclear SREBPs (Figure 6F). Within 0.5 hr after adding 25-HC, the amount of Insig-1 protein had risen detectably, Scap became visible in the Insig-1 immunoprecipitate, and the amount of nuclear SREBP-2 had begun to decline. Insig-1 mRNA remained high. By 1 hr, Insig-1 protein reached a peak, as did the amount of Scap in the Insig-1 immunoprecipitate. Nuclear SREBP-2 was reduced by more than 90%. The fall in Insig-1 mRNA levels lagged behind the drop in nuclear SREBP-2, apparently owing to the half-life of the mRNA, but by 3 hr the Insig-1 mRNA had declined by more than 90%. Despite this decline in mRNA, the Insig-1 protein was accumulating, presumably owing to its stabilization in the Scap/Insig-1 complex. At the conclusion of the experiment (4 hr), the level of Insig-1 protein was the same as it was at zero time even though Insig-1 mRNA had declined by 95%.

**Discussion**

The data in this paper, together with previous data from this laboratory, establish a model for the control of cholesterol supply that we call “convergent feedback inhibition.” The essential elements are diagrammed in Figure 7. In sterol-depleted cells, cholesterol dissociates from the Scap/SREBP complex, and this causes Insig-1 to dissociate as well. The liberated Insig-1 undergoes ubiquitination at lysines 156 and 158, which leads to proteosomal degradation. The liberated Scap/SREBP complex binds to COPII proteins, which cluster it into COPII-coated vesicles for transport to the Golgi. In the Golgi, the SREBP is processed by proteases to release the transcriptionally active NH2-terminal domain, which moves to the nucleus, where it activates transcription of all of the genes that encode enzymes required for cholesterol synthesis and for uptake through the LDL receptor (Horton et al., 2002). As a result, the cell synthesizes cholesterol and takes it up from LDL to correct the deficit. At the same time, nuclear SREBP enhances transcription of the Insig-1 gene, and the resultant Insig-1 protein is inserted into the ER membrane. The newly synthesized Insig-1 will be degraded rapidly unless the cell has accumulated sufficient cholesterol to alter the conformation of Scap so that it binds Insig-1,
forming a stable complex that remains in the ER and resists ubiquitination and degradation. The process is convergent because SREBP processing will not be blocked unless two SREBP products converge on Scap simultaneously: (1) newly synthesized Insig-1 and (2) newly acquired cholesterol. This requirement for convergence assures that SREBP processing will not be terminated prematurely before the SREBP has acted in the nucleus to increase Insig-1 mRNA and to replenish cell cholesterol levels.

Although ubiquitination is required for rapid Insig-1 degradation, it is not required for release of the Scap/SREBP complex. Thus, upon severe sterol depletion, the Scap/SREBP complex is released even when the cells express a mutant Insig-1 that is not ubiquitinated or degraded (Figure 5B). This result is different from that for NF-κB, a soluble transcription factor that is held out of the nucleus by binding to one of several IκB proteins. Nuclear entry of NF-κB occurs only after IκB has been ubiquitinated and degraded (Hoffmann et al., 2002; Chen, 2005).
The essential feature of the model is that SREBP processing will not be blocked by sterols unless two SREBP-induced products converge on Scap simultaneously: (1) newly synthesized Insig-1 and (2) newly synthesized or acquired cholesterol. This convergence assures that SREBP processing will not be terminated before the cholesterol needs of the cell are met.

In order for the convergent feedback mechanism of cholesterol control to operate, two conditions must be met: (1) Insig-1 must be destroyed after sterol depletion, and (2) SREBPs must activate the synthesis of replacement Insig-1 molecules as well as the genes supplying cholesterol. Destruction of Insig-1 creates a requirement for newly synthesized Insig-1, which cannot occur unless sufficient SREBP molecules have entered the nucleus. Thus, sterol synthesis will not be suppressed unless SREBPs have acted on two processes: (1) cholesterol supply through de novo synthesis and uptake via LDL receptors and (2) Insig-1 gene transcription.

The significance of the two components of the Insig-1 control mechanism is underscored by a comparison with Insig-2. In contrast to Insig-1, Insig-2 is neither rapidly degraded nor a transcriptional target of SREBPs. The fact that Insig-1 uniquely possesses both attributes emphasizes the necessity for rapid, sterol-regulated degradation coupled to transcriptional activation in order for the convergent control mechanism to operate.

If Insig-2 does not participate in convergent feedback, what is its role? Studies of mutant CHO cells suggest that Insig-2 functions in long-term control of SREBP processing. Thus, SRD-14 cells lack Insig-1 as a result of loss of function of both alleles of the Insig-1 gene, but they express normal amounts of Insig-2 protein (Sever et al., 2004). In SRD-14 cells, 25-HC fails to block SREBP processing acutely within 5 hr after exposure. However, after 14 hr, SREBP-2 processing is blocked. Thus, at normal levels of expression, Insig-2 cannot replace Insig-1 in achieving short-term control of SREBP processing. The long-term function of Insig-2 may help to smooth out oscillations in SREBP processing that might otherwise occur as Insig-1 levels rise and fall through the convergent feedback mechanism. This would be analogous to the roles of the several isoforms of IκB, a family of proteins that play roles in the NFκB pathway (Hoffmann et al., 2002) that are similar to those of Insigs in the SREBP pathway. Insig-2 may play a more important role than Insig-1 in the liver, where higher amounts of Insig-2 are produced through the action of a liver-specific promoter that is downregulated by insulin (Yabe et al., 2003).

The ubiquitination and degradation of Insig-1 in sterol-deprived cells stand in striking contrast to the Insig-1-dependent ubiquitination and degradation of the cholesterol biosynthetic enzyme HMG CoA reductase that occurs only in sterol-overloaded cells. DeBose-Boyd and coworkers have shown that sterols induce the binding of ER-embedded HMG CoA reductase to Insig-1, and this interaction induces the ubiquitination and degradation of the reductase (Sever et al., 2003). Recently, they have shown that ubiquitination is mediated by gp78, a membrane bound ubiquitin transferase (E3) that forms a complex with Insig-1 (Song et al., 2005). In the presence of sterols, this bound gp78 ubiquitinates HMG CoA reductase. It is tempting to speculate that in the absence of sterols, when neither Scap nor HMG CoA reductase is bound to Insig-1, gp78 ubiquitinates Insig-1, accelerating its degradation. When sterols induce HMG CoA reductase to bind to Insig-1, the ubiquitination reaction is diverted toward HMG CoA reductase. On the other hand, when sterols induce Scap to bind to Insig-1, the gp78 no longer ubiquitinates Insig-1. Perhaps Scap displaces gp78 from Insig-1 in a sterol-dependent reaction.

Both Scap and HMG CoA reductase bind to Insigs in a sterol-dependent fashion by virtue of their shared sterol-sensing domains, which comprise transmembrane helices 2–6 in both proteins. Other proteins have conserved sterol-sensing domains, including Patched (the receptor for the cholesterol-modified morphogen Hedgehog), the Neimann-Pick C1 protein (NPC1) (which functions in intracellular cholesterol transport), and the Neimann-Pick C1-like protein (NPC1L1) (which functions in the intestinal absorption of cholesterol) (Kuwabara and Labouesse, 2002; Altmann et al., 2004). Whether these proteins interact with Insigs in a sterol-dependent manner so as to affect their stability is currently unknown, but the issue should be explored.

**Experimental procedures**

**Materials and antibodies**

These sections are described in the Supplemental Data.

**Plasmid constructs**

The following recombinant expression plasmids have been described: pEF 1aHA-ubiquitin (provided by Dr. Zhijian Chen, University of Texas Southwestern Medical Center), encoding amino acids 1–76 of human ubiquitin preceded by an epitope tag derived from the influenza hemagglutinin (HA) protein (YPYDVPDY) under control of the EF1α promoter (Kanayama et al., 2004); pCMV-Insig1-T7, encoding human Insig-1 followed by three copies of a T7 epitope tag [MASMTGGQMG] under control of the cytomegalovirus (CMV) promoter (Song et al., 2005); pCMV-Scap, encoding wild-type hamster Scap under control of the CMV promoter (Sakai et al., 1997); and pTK-Scap, recoding wild-type and mutant hamster Scap, respectively, under control of the thymidine kinase (TK) promoter (Nohrufft et al., 1998).

pTK-Insig1-Myc encoding wild-type human Insig-1 followed by six tandem copies of a c-Myc epitope tag (EQKLISEEDL) under control of the TK promoter was generated as described in the Supplemental Data. pTK-Insig1[K156R/K158R]-Myc and pCMV-Insig1[K156R/K158R]-T7 were generated by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The coding regions of all plasmids were sequenced before use.

**Tissue culture media**

Medium A contained a 1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin and
100 μg/ml streptomycin sulfate. Medium B contained medium A supplemented with 5% fetal calf LPDS, 50 μM sodium compactin, and 50 μM sodium mevalonate. Medium C contained medium A supplemented with 5% fetal calf serum, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μM sodium oleate. Medium D contained methionine- and cysteine-free RPMI 1640 medium supplemented with 5% newborn calf LPDS, 50 μM sodium compactin, and 50 μM sodium mevalonate.

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 LPDS (Metherall et al., 1989). SRD-13A cells (Scap−/−) (Rawson et al., 1999) and SRD-14 cells (Insig-1−/−) (Sever et al., 2004) are mutant cells derived from CHO-7 cells. SRD-14/pTK-Insig1-Myc cells are a clone of SRD-14 cells stably transfected with pTK-Insig1-Myc (see below). SRD-14/pTK-Insig1(K156R/K158R)-Myc cells are a clone of SRD-14 cells stably transfected with pTK-Insig1(K156R/K158R)-Myc (see below).

Stock cultures of CHO-7 cells were maintained in medium A supplemented with 5% newborn calf LPDS. SRD-14 cells were maintained in medium C. SRD-14/pTK-Insig1-Myc cells and SRD-14/pTK-Insig1(K156R/K158R)-Myc cells were maintained in medium A supplemented with 5% LPDS and 500 μg/ml G418.

Transient transfection of cells
SRD-13A cells were transiently transfected with FuGENE 6 reagent (Roche Diagnostics) according to the manufacturer’s protocol. The total amount of DNA in each transfection was adjusted to 3 μg per dish by addition of pcDNA3 mock vector. Conditions of incubation after transfection are described in the Figure legends. At the end of the incubation, duplicate dishes of cells for each variable were harvested and pooled for analysis.

Stable transfection of cells
SRD-14/pTK-Insig1-Myc and SRD-14/pTK-Insig1(K156R/K158R)-Myc cells, derivatives of SRD-14 cells, were generated as follows. On day 0, SRD-14 cells were set up at 5 × 10⁶ cells per 100 mm dish in medium A with 5% LPDS. On day 1, the cells were transfected with 1 μg of pTK-Insig1-Myc or pTK-Insig1(K156R/K158R)-Myc using the FuGENE 6 transfection reagent as described above. On day 2, the cells were switched to medium A supplemented with 5% LPDS and 700 μg/ml G418. Fresh medium was added every 2–3 days until colonies formed after about 2 weeks. Individual colonies were isolated with cloning cylinders, and Insig-1 expression was assessed by immunoblot analysis with anti-Myc. Cells were chosen based on their transgene expression such that Insig-1 expression level in SRD-14/pTK-Insig1-Myc cells is equal to that in SRD-14/pTK-Insig1(K156R/K158R)-Myc cells in the presence of 1 μg/ml of 25-HC. Cells from single colonies were cloned by limiting dilution and maintained in medium A containing 5% LPDS and 500 μg/ml G418.

Immunoblot analysis, immunoprecipitation, and real-time PCR
These methods are described in the Supplemental Data.

Pulse-chase analysis in CHO-7 cells
CHO-7 cells were incubated in medium B as described above. Sixteen hours after incubation, the cells were washed with phosphate-buffered saline and switched to methionine- and cysteine-free medium D. After 1 hr at 37°C, the cells were pulse labeled with 200 μCi/ml [35S]Protein Labeling Mix (Perkin-Elmer Life Sciences) in 1.5 ml of medium D. After labeling for 1 hr, the medium was removed, and the cells were washed twice with phosphate-buffered saline and chased for various times in medium B (containing 0.12 mM unlabeled methionine and 0.4 mM unlabeled cysteine) in the absence or presence of 1 μg/ml 25-HC. Following the chase, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline and lysed in 1 ml of buffer B (50 mM Tris-Cl [pH 7.2], 150 mM NaCl, 0.5% [v/v] Fos-10, and protease inhibitor cocktail). The lysates were centrifuged at 20,000 × g for 20 min at 4°C. The supernatants (1 ml) were transferred to fresh tubes and preclarified by rotation for 16 hr at 4°C with 50 μl of protein A/G agarose (Santa Cruz) and 20 μg/ml control nonimmune IgG. After centrifugation at 300 × g for 7 min at 4°C, the supernatants were transferred to fresh tubes, and 10 μl of polyclonal antiserum against Insig-1 (1:100 dilution) together with 50 μl of protein A/G agarose beads was added and rotated for 2 hr at 4°C. The beads were collected by centrifugation, washed six times (30 min each) in 1 ml of buffer B, resuspended in 50 μl of 1× SDS loading buffer, and boiled for 5 min. After centrifugation at 16,000 × g for 5 min at room temperature, the supernatants were transferred to fresh tubes, 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 24 hr, and scanned in a Molecular Dynamics Storm 820 phosphorimaging device.

Ubiquitination of Insig-1
Cells were lysed in 0.1 ml of buffer B supplemented with 10 mM N-ethylmaleimide, rotated at 4°C for 1 hr, and centrifuged at 100,000 × g for 15 min at 4°C. The resulting supernatants were mixed with 0.3 ml of 8 M urea in buffer B, rotated for 10 min at room temperature, diluted with buffer B to decrease the urea concentration to 2 M, and subjected to immunoprecipitation (16 hr at 4°C) with 50 μl of monoclonal anti-T7 IgG-coupled agarose beads (Novagen) directed against the T7 epitope tag on Insig-1, followed by low-speed centrifugation. The resulting supernatants were mixed with 5× SDS loading buffer, and the pelleted beads were washed three times (20 min each) with buffer B and resuspended in 50 μl of 1× SDS loading buffer. The supernatant and pellet fractions were boiled for 5 min and subjected to SDS-PAGE and immunoblotting with an antibody against the HA tag on ubiquitin.

Supplemental data
Supplemental Data include Supplemental Experimental Procedures and Supplemental References and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/1/15/DC1/.

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