Insig-1 and Insig-2 are membrane proteins of the endoplasmic reticulum that regulate lipid metabolism by the following two actions: 1) sterol-induced binding to 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an action that leads to ubiquitination and degradation of the enzyme; and 2) sterol-induced binding to SREBP cleavage-activating protein, an action that blocks the proteolytic processing of sterol regulatory element-binding proteins (SREBPs), membrane-bound transcription factors that enhance the synthesis of cholesterol and fatty acids. Here we report the isolation of a new mutant line of Chinese hamster ovary cells, designated SRD-14, in which Insig-1 mRNA and protein are not produced due to a partial deletion of the INSIG-1 gene. The SRD-14 cells were produced by γ-irradiation, followed by selection with the 1,1-bisphosphonate ester SR-12813, which mimics sterols in accelerating reductase degradation but does not block SREBP processing.

SRD-14 cells fail to respond to sterols by promoting reductase ubiquitination and degradation. The rate at which sterols suppress SREBP processing is significantly slower in SRD-14 cells than wild type CHO-7 cells. Sterol regulation of reductase degradation and SREBP processing is restored when SRD-14 cells are transfected with expression plasmids encoding either Insig-1 or Insig-2. These results provide formal genetic proof for the essential role of Insig-1 in feedback control of lipid synthesis in cultured cells.

Insig-1 and Insig-2, a pair of closely related endoplasmic reticulum (ER)-localized membrane proteins, play a substantial role in the feedback control of lipid synthesis in animal cells. Insigs coordinate lipid synthesis via their sterol-dependent binding to two polytopic ER membrane proteins: sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). Sterol-dependent binding of Insigs to SCAP leads to the ER retention of SREBP complexes, thus preventing their translocation to the Golgi where the SREBPs are activated by sequential proteolytic cleavages (1, 2). Sterol-induced binding of Insigs to reductase leads to its ubiquitination and consequent degradation by proteasomes, thus slowing the rate-limiting reaction in cholesterol synthesis (3, 4). The two human Insig proteins are 59% identical and are predicted to contain six membrane-spanning helices (5). Insig-1 and Insig-2 appear to have overlapping functions in mediating sterol regulation of SREBP and reductase, but the proteins differ in their mode of regulation (2, 6). In cultured cells, the INSIG-1 gene is a target of SREBPs, and its mRNA rises and falls according to the nuclear content of processed SREBPs. The INSIG-2 gene is controlled by two promoters that give rise to alternative transcripts, designated Insig-2a and Insig-2b, with different noncoding first exons spliced into a common second exon. The Insig-2b transcript is ubiquitous and, in cultured cells, is unvarying; it is not influenced by SREBPs. The Insig-2a transcript is expressed exclusively in the liver and is negatively regulated by insulin.

SREBPs are a family of membrane-bound transcription factors that enhance transcription of genes encoding cholesterol and fatty acid biosynthetic enzymes and the LDL receptor (7). Newly synthesized SREBPs localize to membranes of the ER and nuclear envelope with a significant fraction of them found in tight association with SCAP. In sterol-depleted cells, SCAP escorts SREBPs to the Golgi where the SREBPs are proteolytically released from membranes, thereby allowing transcriptionally active fragments to enter the nucleus and modulate target gene transcription. Sterols inhibit SREBP activation by preventing the exit of SREBP-SCAP complexes from the ER, leading to decreased synthesis of cholesterol and other lipids (8). The block in SREBP export is achieved through sterol-induced binding of SCAP to Insigs (1, 2, 9). The Insig-binding site in SCAP is located within its membrane domain, which comprises eight membrane-spanning segments (10). A segment corresponding to helices 2–6 of the SCAP membrane domain demonstrates significant sequence similarities to the corresponding region in reductase, whose membrane domain also contains eight membrane-spanning segments (11), and this region has become known as the sterol-sensing domain (12–14). Point mutations within the sterol-sensing domain of SCAP prevent it from binding Insigs (1, 2), thus preventing sterol-mediated ER retention of SREBP complexes (8, 9, 15, 16).

HMG-CoA reductase produces mevalonate, an important intermediate in the synthesis of cholesterol and nonsterol isoprenoids (17). Sterol and nonsterol end products of mevalonate metabolism accelerate degradation of reductase as part of a complex, multivalent feedback mechanism that contributes to sterol homeostasis (18). When reductase is overexpressed in

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Chinese hamster ovary (CHO) cells by transfection, the enzyme is no longer rapidly degraded in response to sterol treatment (3). Overexpression of Insig-1 restores regulated degradation of overexpressed reductase, and this degradation can be inhibited by overexpressing the sterol-sensing domain of SCAP, suggesting the two proteins compete for limiting amounts of Insigs when sterol levels are high. The combined knockdown of Insig-1 and Insig-2 through RNA interference (RNAi) abolishes sterol-regulated ubiquitination and subsequent degradation of endogenous reductase in human fibroblasts (4). Ubiquitination and degradation of reductase require its sterol-dependent binding to Insigs, as indicated by the observation that point mutations within the reductase sterol-sensing domain prevent its association with Insigs, rendering the enzyme resistant to sterol-stimulated ubiquitination and degradation. Although the sterol effects on reductase and SCAP require binding to Insigs, the consequences appear remarkably different. After binding to Insigs, reductase is degraded by a process mediated by ubiquitin-proteasomal degradation, whereas binding of SCAP to Insigs leads to its ER retention. To understand how binding to Insigs can have such different effects on its two ligands requires the development of procedures through which other members of the SCAP-Insig and reductase-Insig complexes can be identified.

Hmg2p, one of two reductase isozymes expressed in the yeast *Saccharomyces cerevisiae*, undergoes regulated degradation that displays similarities to the analogous process in mammalian cells (19). The molecular participants in regulated degradation of Hmg2p have been revealed through genetic analyses of *HRD* (HMG-CoA Reductase Degradation) mutants (20). *HRD* genes encode proteins that play distinct roles in the Hmg2p degradative pathway (21). For example, the *HRD1* and *HRD3* genes encode proteins that constitute a membrane-bound ubiquitin ligase complex that mediates Hmg2p ubiquitination in response to degradative signals (22). The *HRD2* gene encodes a subunit of the 26 S proteasome, supporting a role for proteasomes in regulated Hmg2p degradation, whereas *HRD4* mutants are defective in post-ubiquitination steps prior to proteasomal destruction of the enzyme (23, 24). Considering this and the successful application of somatic cell genetics in unraveling the SREBP pathway (25), a genetic analysis of mammalian reductase degradation should expedite the identification of molecules recruited to the sterol-dependent reductase-Insig complex. However, in order to use genetic complementation and expression cloning techniques like those used for the identification of SCAP and the proteases that release SREBPs from membranes, mutant cells defective in sterol-activated reductase degradation are required.

In the past, mutated CHO cells were cultured in the continual presence of 25-hydroxycholesterol, a potent inhibitor of cholesterol synthesis because of its ability to accelerate degradation of reductase and block proteolytic activation of SREBPs (8, 13, 26). The oxysterol is toxic to wild type cells because it cannot substitute for cholesterol as a structural membrane component, and cells die unless a usable source of cholesterol is supplied exogenously. Mutant cells survive chronic 25-hydroxycholesterol treatment because they do not turn off cholesterol synthesis in the presence of oxysterols, forming the genetic basis for their isolation. However, the sterol-resistant mutants isolated to date either harbor point mutations in the sterol-sensing domain of SCAP or produce abnormal truncated versions of SREBP-2 that enter the nucleus without requiring proteolysis (9, 12, 27–29). Thus, to facilitate the isolation of mutant cells incapable of accelerating reductase degradation, selection of mutated cells must be carried out with a reagent that replaces sterols to promote reductase degradation but not ER retention of SCAP.

The hypocholesterolemic activity of 1,1-bisphosphonate esters such as SR-12813, and its structurally related analog, Apomine, has been well documented (30–33). In cultured cells, these drugs decrease reductase activity by accelerating degradation of the enzyme which triggers an increase in the expression of LDL receptors, thus explaining the hypocholesterolemic activity of the drugs when administered orally in vivo. The effects of SR-12813 on reductase appear similar to those of oxysterols; however, unlike oxysterols, SR-12813 treatment does not reduce but rather enhances mRNAs for reductase and the LDL receptor.

In the current studies, we evaluated the utility of SR-12813 as a selecting agent in mutagenesis experiments aimed toward isolating mutant cells incapable of accelerating reductase degradation. The resultant cell line, designated SRD-14, was found to harbor a partial deletion of the *INSIG-1* gene. SRD-14 cells cannot accelerate reductase degradation in response to sterols or SR-12813, and the rate of sterol-mediated suppression of SREBP processing in the cells is dramatically reduced. The experiments with the SRD-14 cells provide formal genetic proof of the essential role Insigs play in not only reductase degradation but also the ER retention of SCAP-SREBP.

**EXPERIMENTAL PROCEDURES**

**MATERIALS—**We obtained MG-132 from Calbiochem; horseradish peroxidase-conjugated donkey anti-mouse, and anti-rabbit IgGs were from Jackson ImmunoResearch; SR-12813 was from Dr. Timothy M. Wilson, GlaxoSmithKline (Research Triangle Park, NC) or synthesized by Sandhya Kulkarni of the Core Medicinal Chemistry Laboratory, Department of Biochemistry, University of Texas Southwestern Medical Center; and sterols (25-hydroxycholesterol and cholesterol) from Steraloids, Inc. (Newport, RI). Other reagents were obtained from sources described previously (34). Lipoprotein-deficient serum (LPDS, d = 1.215 g/ml) was prepared from newborn calf serum by ultracentrifugation (35). The following recombinant plasmids were described previously in the indicated reference: pCMV-Insig-1-Myc, encoding amino acids 1–277 of human Insig-1 followed by six tandem copies of a c-Myc epitope tag under control of the cytomegalovirus (CMV) promoter (1); pCMV-Insig-2-Myc, encoding amino acids 1–225 of human Insig-2 followed by six tandem copies of a c-Myc epitope tag under control of the CMV promoter (2); pCMV-HMG-Red-T7, encoding amino acids 1–887 of hamster HMG-CoA reductase followed by three tandem copies of the T7 epitope tag under control of the CMV promoter (3); pCMV-HMG-Red-T7, encoding amino acids 1–346 of hamster HMG-CoA reductase followed by three tandem copies of the T7 epitope tag under control of the CMV promoter (3). The lysing (K98R/K248R) and sterol sensing domain (Y75F/F75F/F75F) mutations of pCMV-HMG-Red-T7 and pCMV-HMG-Red-T7 (TM 1–8) were described previously (4).

**Cell Culture—**Monolayers of SV-588 cells, an immortalized line of human fibroblasts expressing the SV40 large T antigen (36), were grown at 37 °C in 5% CO2. Stock cultures of SV-588 cells were maintained in medium A (Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) fetal calf serum (FCS).

CHO-7 cells, a subline of CHO-K1 cells selected for growth in LPDS (37), were grown in monolayer at 37 °C in 8–9% CO2. The cells were maintained in medium B (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% LPDS.

Monolayers of UT-2 cells, a line of mutant CHO cells that lacks HMG-CoA reductase (38), were grown at 37 °C in 8–9% CO2. Stock cultures of UT-2 cells were maintained in medium B supplemented with 5% LPDS and 200 μM mevaquin.

**Transient Transfection—**Transfections were performed as described (39) with minor modifications. CHO-7 cells were transfected with 3 μg of DNA per 60-mm dish. For each transfection, FuGENE-6 DNA transfection reagent (Roche Diagnostics) was added to 0.2 ml of medium B at a ratio of 3 μl of FuGENE-6 per 1 μg of DNA. Conditions of the incubations are described in the figure legends. At the end of the incubations, triplicate dishes of cells for each variable were harvested and pooled for analysis.

**Cell Fractionation and Immunoblot Analysis—**The pooled cell pellets from triplicate dishes of cells were used to isolate 2 × 10^7 × g membrane
fractions and/or nuclear extract fractions as described previously (3) with a minor modification. The nuclear extract fractions (300 μl) were precipitated with 1.5 ml of acetone for 16 h at −20 °C. Precipitated material was collected by centrifugation at 2 × 105 × g for 15 min and solubilized in 100 μl of buffer containing 10 mM Tris-HCl (pH 8.5), 1% (w/v) SDS, 100 mM NaCl, 1 mM EDTA, and 1 mM EGTA, mixed with 25 μl of 5 × SDS loading buffer, and boiled for 5 min. Aliquots of nuclear extract and membrane fractions were subjected to 8% SDS-PAGE, and immunoblot analysis was carried out as described (3). Primary antibodies used for immunoblotting are as follows: mouse monoclonal anti-T7-Tag (IgG2b) (Novagen); mouse monoclonal anti-Myc (IgG fraction) from the culture medium of hybridoma clone 9E10 (American Type Culture Collection); IgG-A9, a mouse monoclonal antibody against the catalytic domain of hamster HMG-CoA reductase (amino acids 456–587) (40); IgG-ID2, a mouse monoclonal antibody against the NH2 terminus of human SREBP-2 (amino acids 48–403) (2); IgG-T7D4, a mouse monoclonal antibody against the NH2 terminus of hamster SREBP-2 (28); anti-Insig-1, a rabbit polyclonal antibody against full-length mouse Insig-1 (41); R-139, a rabbit polyclonal antibody against amino acids 54–207 and 540–707 of hamster SCAP (42); and IgG-P4D1, a mouse monoclonal antibody against bovine ubiquitin (Santa Cruz Biotechnology).

Ubiquitination of HMG-CoA Reductase—Conditions of incubations are described in the figure legends. At the end of the incubations, the cells were harvested and lysed in detergent-containing buffer, and immunoprecipitation of reductase from the detergent lysates was carried out with polyclonal antibodies against the 60-kDa COOH-terminal domain of human reductase as described previously (3, 4, 43). Aliquots of the immunoprecipitates were subjected to SDS-PAGE on 6% gels, transferred to nylon membranes, and subjected to immunoblot analysis.

RNA Interference—Duplexes of small-interfering RNA targeting human Insig-1, human Insig-2, and an irrelevant control gene, vesicular stomatitis virus glycoprotein (VSV-G), were synthesized by Dharmacon Research (Lafayette, CO), and RNAi experiments were carried out as described previously (4). Mutagenesis and Isolation of SR-12813-resistant Cells Deficient in Insig-1—On day 0, 2.5 × 106 CHO-7 cells were subjected to γ-irradiation as described previously (44). The cells were immediately plated at 105 cells/100-mm dish in medium B supplemented with 5% LPDS. On day 1, the medium was replaced with medium B containing 5% LPDS and 10 μg SR-12813. Fresh medium was added to the cells every 2 days until colonies formed. On day 29, the surviving colonies were isolated with cloning cylinders and allowed to proliferate. Of the 50 original dishes, 14 contained SR-12813-resistant colonies, and it was determined that 8 of the colonies lacked expression of Insig-1 mRNA. The most vigorous colony was cloned by limiting dilution and designated SRD-14 cells.

SRD-14/pInsig-1 and SRD-14/pInsig-2 cells, derivatives of SRD-14 cells stably expressing Insig-1-Myc or Insig-2-Myc, respectively, were generated as follows. On day 0, SRD-14 cells were set up at 5 × 106 cells per 60-mm dish in medium B supplemented with 5% LPDS. On day 1, the cells were transfected with 1 μg of pCMV-Insig-1-Myc or pCMV-Insig-2-Myc using the FuGENE 6 transfection reagent as described above. On day 2, cells were switched to medium B 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 or Insig-2 expression was assessed by immunoblot analysis with anti-Myc. Cells from a single colony were cloned by limiting dilution and maintained in medium B containing 5% LPDS and 500 μg/ml G418 at 37 °C, 8–9% CO2.

Real Time PCR and Northern and Southern Blot Analysis—The protocol for real time PCR was identical to that described by Liang et al. (45). Total RNA was isolated from CHO-7 and SRD-14 cells using the RNeasy kit (Qiagen) according to the manufacturer’s instructions and subjected to reverse transcription. Triplicate samples of reverse-transcribed total RNA were subjected to real time PCR quantification using forward and reverse primers for hamster HMG-CoA reductase, HMG-CoA synthase, LDL receptor, Insig-1, Insig-2, and glyceroldehyde-3-phosphate dehydrogenase (invariant control) (Table I). Relative amounts of mRNAs were calculated using the comparative Ct method. Hamster Insig-1 cDNA probes for Northern and Southern blot analysis were prepared by PCR amplification of reverse-transcribed total RNA isolated from wild type CHO-7 cells using the following forward and reverse primers: 5′-CCAGGATGCATGCGCCAGTGAAGG-3′ and 5′-CCAGGCCGCGCCGCTAGCTGAGGTTTTCGGCCG-3′. The resulting PCR products, encoding the entire coding region of Insig-1, were radiolabeled with [α-32P]dCTP using the Megaprime DNA Labeling System (Amersham Biosciences). Total RNA and restriction enzyme-digested genomic DNA were subjected to electrophoresis and transferred to Hybond N+ membranes (Amerham Biosciences), and filters were hybridized at 60–65 °C with radiolabeled probe (2 × 106 cpm/ml and 4 × 105 cpm/ml for Northern and Southern blots, respectively) using the ExpressHyb Hybridization Solution (Clontech) according to the manufacturer’s instructions. Filters were exposed to film with intensifying screens for the indicated time at ~80 °C.

RESULTS

The experiments shown in Fig. 1 were designed to determine whether SR-12813 utilizes mechanisms distinct from those of steroids to accelerate degradation of reductase. SV-S89 cells, a line of transformed human fibroblasts, were depleted of sterols by incubation for 16 h in medium containing LPDS, the reductase inhibitor, compactin (46), and a low level of mevalonate (50 μM), which is the lowest level that ensures viability (Fig. 1A). Cells were then treated for an additional 5 h with various combinations of SR-12813, a high level of mevalonate (10 mM), and 30 μM geranylgeraniol (the alcohol derivative of geranylgeranyl pyrophosphate). Following treatments, the cells were harvested and separated into membrane and nuclear extract fractions. Aliquots of the fractions were subsequently subjected to SDS-PAGE and immunoblotted with anti-reductase and anti-SREBP-2 monoclonal antibodies. In untreated cells, we observed a full-length band of reductase that remained unchanged upon treatment of the cells with SR-12813 alone (Fig. 1A, top panel, lanes 1 and 2). However, when cells were treated with the combination of SR-12813 and mevalonate or geranylgeraniol, reductase degradation was accelerated as indicated by the dramatic decrease in the reductase band (Fig. 1A, top panel, lanes 4 and 6, respectively). These results indicate that nonsterol mevalonate-derived products or geranylgeraniol can synergize with SR-12813 to accelerate degradation of reductase. Similarly, we and others (4, 47, 48) have observed that these nonsterol products of mevalonate metabolism also significantly contribute to sterol-activated degradation of reductase. In contrast to its effects on the degradation of reductase, SR-12813 did not alter SCAP activity, as indicated by the persistence of nuclear SREBP-2, regardless of treatment conditions (Fig. 1A, bottom panel, lanes 1–6). These results

| mRNA | Sequences of forward and reverse primers (5′–3′) | GenBank™ accession no. |
|------|-------------------------------------------------|------------------------|
| Insig-1 | GGCTTGTGGTGGACAATTG | AF527682 |
| Insig-2 | GGCTTGTGGTGGACCTCTCATG | AF527692 |
| HMG-CoA reductase | AGATACTGGAGTGGCAGAAAA | X00494 |
| HMG-CoA synthase | TTGTAGCTGGATGTGGCTTT | AH016829 |
| LDL receptor | AGACATAGCGACGAGAATGAG | M13877 |
| Glyceraldehyde-3-phosphate dehydrogenase | GACCCATCTTCGCTGGCAATA | XS2123 |

TABLE I

Nucleotide sequences of hamster-specific primers used for quantitative real time PCR
SR-12813 mimics sterols in promoting Insig-dependent ubiquitination and degradation of HMG-CoA reductase and cannot accelerate degradation-resistant forms of the enzyme. A, SV-589 cells were set up for experiments on day 0 at 2 × 10⁶ cells per 100-mm dish in medium A containing 10% FCS. On day 2, the cells were washed with phosphate-buffered saline and refed medium A supplemented with 10% LPDS, 50 μM sodium compactin, and 50 μM sodium mevalonate. After 16 h at 37°C, cells were switched to medium A containing 10% LPDS and 50 μM compactin in the absence (−) or presence (+) of 10 μM SR-12813, 10 mM mevalonate, and 30 μM geranylgeraniol (GG-OH), as indicated. After incubation at 37°C for 5 h, the cells were harvested and subjected to cell fractionation as described under “Experimental Procedures.” Aliquots of the membrane (3 μg of protein/lane) and nuclear extract fractions (30 μg of protein/lane) were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblotted with 5 μg/ml monoclonal IgG-A9 (against reductase) or 5 μg/ml monoclonal IgG-1D2 (against human SREBP-2). Filters were exposed to film for 10 s at room temperature. B and C, SV-589 cells were set up on day 0 at 5 × 10⁴ cells per 60-mm dish in medium A with 10% FCS. On days 1 and 3, cells were transfected with 400 pmol/dish of VSV-G siRNA (lanes 1–3) or Insig-1 and Insig-2 siRNA (lanes 4–6) as described under “Experimental Procedures.” After the second transfection on day 3, cells were incubated for 16 h at 37°C in medium A containing 10% LPDS, 50 μM compactin, and 50 μM mevalonate. On day 4, the cells were switched to medium A containing 10% LPDS, 50 μM compactin, and 10 mM mevalonate in the absence (−) or presence (+) of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) or 10 μM SR-12813, as indicated. C, the medium also contained 10 mM MG-132. B, after 5 h at 37°C, the cells were harvested and subjected to SDS-PAGE and immunoblot analysis as in A. Filters were exposed to film for 5 s. C, after 1 h at 37°C, cells were harvested, lysed, and subjected to immunoprecipitation with polyclonal anti-reductase antibody (lanes 1–3) or 0.2 μg/ml IgG-P4D1 (against ubiquitin). Filters were exposed to film for 15 (top gel) and 30 s (bottom gel). D, CHO-7 cells were set up on day 0 at 5 × 10⁵ cells per 60-mm dish in medium B with 5% LPDS. On day 1, cells were transfected in 2 ml of medium A containing 5% LPDS and 1 μg of wild type (lanes 1–4), K89R/K248R (lanes 5–8), or Y1YF to AAAA (lanes 9–12) versions of pCMV-HMG-Red-T7 (TM 1–8) in the absence or presence of 20 ng of pCMV-Insig-1-Myc as indicated. The total amount of DNA in each lane was adjusted to 5 μg/dish by the addition of pcDNA3 empty vector. Six hours after transfection, cells received a direct addition of 2 ml of medium B containing 5% LPDS, 10 μM compactin, and 50 mM mevalonate (final concentrations). After 16 h at 37°C, cells were switched to medium B containing 5% LPDS and 50 μM mevalonate in the absence (−) or presence (+) of 10 μM SR-12813 plus 10 mM mevalonate as indicated. After incubation for 5 h at 37°C, cells were harvested, membrane fractions prepared, and aliquots (5 μg of protein/lane) were subjected sequentially to SDS-PAGE and immunoblot analysis with 1 μg/ml anti-T7 IgG (against reductase) or 1 μg/ml monoclonal anti-Myc IgG (against Insig-1). Filters were exposed to film for 1–10 s.

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indicate that SR-12813 can replace sterols to promote accelerated degradation of reductase, but the drug cannot substitute for sterols in mediating ER retention of the SCAP-SREBP-2 complex. It should be noted that in this and subsequent experiments in this report, regulation of nuclear SREBP-1 mirrored that of nuclear SREBP-2 (data not shown).

In previous studies, we used RNAi to demonstrate that sterol-dependent degradation and ubiquitination of endogenous reductase requires either Insig-1 or Insig-2 (4). To determine whether Insigs are required for SR-12813-dependent degradation and ubiquitination of reductase, SV-589 cells were transfected with duplexes of small interfering RNA (siRNA) targeting the control gene, vesicular stomatitis virus glycoprotein, which is not expressed in the cells, or the combination of Insig-1 and Insig-2. For degradation experiments (Fig. 1B), cells were treated for 5 h with 10 mM mevalonate and either sterols or SR-12813. In control transfected cells, sterols and SR-12813 accelerated the degradation of reductase (Fig. 1B, lanes 1–3), yet when siRNAs targeting Insig-1 and Insig-2 were introduced into the cells, both sterol- and SR-12813-dependent degradation of reductase was severely blunted (lanes 4–6). Similar results were obtained for reductase ubiquitination (Fig. 1C). For ubiquitination experiments, the cells were treated for 1 h with 10 mM mevalonate, the proteasome inhibitor MG-132 (to prevent degradation of ubiquitinated reductase), and either sterols or SR-12813. Following the incubation, the cells were harvested, lysed in detergent-containing buffer, and subjected to immunoprecipitation with polyclonal anti-reductase antibody. Sterols and SR-12813 stimulated ubiquitination of reductase to similar levels in the control transfected cells, as indicated by the appearance of high molecular weight smears in anti-ubiquitin immunoblots of the reductase immunoprecipitates (Fig. 1C, lanes 1–3). Ubiquitination of reductase was ablated when the cells received Insig-1 and Insig-2 siRNAs (Fig. 1C, lanes 4–6). Together, the results of Fig. 1, B and C, demonstrate that sterols and SR-12813 promote ubiquitination and degradation of reductase through a shared Insig-dependent mechanism.

When overexpressed in CHO cells, we previously found that reductase was no longer rapidly degraded in response to sterols.
Accelerated degradation of reductase was restored by over-expressing Insig-1 or Insig-2, and by using this assay, we have identified amino acid residues in reductase required for accelerated degradation of the enzyme (4). Alanine substitution experiments revealed that binding of reductase to Insigs and its subsequent ubiquitination and degradation required a tetrapeptide sequence, YIYF, in the reductase sterol-sensing domain. The YIYF peptide also appears in the sterol-sensing domain of SCAP, where it mediates formation of the sterol-dependent SCAP-Insig complex (1, 2). In contrast, the conservative substitution of arginine for lysines 89 and 248 of reductase did not alter Insig binding, but reductase was no longer ubiquitinated, and degradation was markedly slowed, implicating the lysine residues as sites for Insig-mediated, sterol-dependent ubiquitination. Thus, we next sought to determine the activity of SR-12813 toward degradation-resistant forms of reductase (Fig. 1D). Given that the membrane domain of reductase is not only necessary but sufficient for regulated degradation (49, 50), we transfected CHO-7 cells with wild type or mutant forms of pCMV-HMG-Red-T7 (TM 1–8), an expression plasmid encoding a truncated version of reductase that contains the entire membrane domain (transmembrane segments 1–8) but lacks the entire catalytic domain. The COOH terminus of this protein is composed of three tandem copies of the T7 epitope tag. Upon overexpression in CHO-7 cells, the wild type reductase membrane domain was not subject to accelerated degradation with SR-12813 treatment (Fig. 1D, top panel, lanes 1 and 2). Likewise, both mutant forms of the reductase membrane domain (K89R/K248R and YIYF to AAAA) were not rapidly degraded when transfected alone (Fig. 1D, top panel, lanes 5, 6, 9, and 10). However, co-transfection of pCMV-Insig-1-Myc, an expression plasmid encoding amino acids 1–277 of human Insig-1 followed by six tandem copies of the c-Myc epitope tag (Fig. 1D, bottom panel), resulted in SR-12813-dependent degradation of wild type but not mutant versions of the reductase membrane domain (top panel, compare lanes 3 and 4 with 7, 8, 11, and 12). These results indicate that, much like the situation with sterols, SR-12813-dependent degradation of the reductase membrane domain requires amino acids implicated in the covalent attachment of ubiquitin (lysines 89 and 248) and binding of the reductase to Insigs (the YIYF tetrapeptide). Additionally, the Insig requirement for SR-12813-dependent degradation of the reductase membrane domain is consistent with the Insig requirement for SR-12813-mediated degradation of full-length, endogenous reductase (Fig. 1). Considering that SR-12813 effectively replaces sterols for Insig-dependent regulation of reductase degradation, but not ER retention of SCAP (see Fig. 1), we reasoned the specificity of SR-12813 could be exploited to isolate mutant cells that no longer accelerate degradation of reductase. The experiment of Fig. 2A, which shows a series of stained Petri dishes, was designed to determine the effects of SR-12813 on the growth of CHO-7 cells. CHO-7 cells are a derivative of CHO-K1 cells that exhibit high rates of cholesterol synthesis because of their selection for growth in LDL, a condition that renders cells dependent upon endogenous cholesterol synthesis for survival (37). The growth of CHO-7 cells tolerated supplementation of LDL-containing medium with as much as 2 μM SR-12813. However, the cells failed to proliferate when challenged with 4–10 μM SR-12813, and growth could be restored by the addition of cholesterol (5 μg/ml) to the culture medium. This indicates that toxicity of SR-12813 is attributable to its ability to accelerate degradation of reductase, thereby inhibiting cholesterol synthesis, and ultimately leading to cell death as a result of cholesterol depletion. To demonstrate its toxic effects are due to accelerated degradation of reductase, we assessed the ability of SR-12813 to kill cells expressing degradation-resistant forms of the enzyme. For this purpose, we transfected the reductase-deficient UT-2 cells (38) with wild type or mutant versions (K89R/K248R and YIYF to AAAA) of pCMV-HMG-Red-T7, an expression plasmid encoding full-length reductase followed by three tandem copies of the T7 epitope tag (4). Following transfection and selection of transformants, the cells were grown in LDL-containing medium with or without SR-12813 supplementation. As shown in Fig. 2B, LDL-containing medium failed to support growth of mock-transfected cells, due to the lack of reductase activity in UT-2 cells. Growth of the cells in LDL-containing medium was restored by the transfection of wild type and mutant forms of reductase, but only those cells expressing the degradation-resistant forms of reductase survived when challenged with SR-12813. Together, the results of Fig. 2 demonstrate that SR-12813 efficiently kills CHO-7 cells as a result of the ability of the drug to promote reductase degradation, which leads to the toxic depletion of cholesterol. Most importantly, these results support our objective of isolating mutant cells that can survive chronic SR-12813 treatment because of their inability to stimulate degradation of reductase. Approximately 2.5 × 10⁷ CHO-7 cells were mutagenized with γ-irradiation and subjected to selection in 10 μM SR-12813. The most vigorous clone was isolated, expanded, and designated SRD-14 (sterol regulatory defective-14) cells. The growth assay in Fig. 3A shows that SRD-14 cells were resistant to growth in 10 μM SR-12813 and up to 0.1 μM/25-hydroxycholesterol. In contrast, the parental CHO-7 cells were effectively killed by SR-12813, and their growth exhibited a greater sensitivity (about 3-fold) to 25-hydroxycholesterol than that of SRD-14 cells. The resistance of SRD-14 cells to SR-12813 and
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Fig. 3. Comparison of growth, HMG-CoA reductase degradation/ubiquitination, and SREBP processing in parental CHO cells and mutant SRD-14 cells treated with sterols and SR-12813. A, CHO-7 and SRD-14 cells were set up on day 0 at 4 × 10⁶ cells per 60-mm dish in medium B containing 5% LPDS. On day 1, the cells were refed medium B supplemented with 5% LPDS and 10 μM SR-12813 or the indicated concentration of 25-hydroxycholesterol. Cells were refed every 2–3 days. On day 14, the cells were washed, fixed in 95% ethanol, and stained with crystal violet. B and C, CHO-7 and SRD-14 cells were set up on day 0 at 5 × 10⁵ cells per 100-mm dish in medium B containing 5% LPDS. On day 2, the cells were refed medium B supplemented with 5% LPDS, 10 μM compactin, and 50 μM mevalonate. After 16 h at 37 °C, cells were switched to medium B containing 5% LPDS and 50 μM compactin in the absence (−) or presence (+) of either sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) or 10 μM SR-12813 plus 10 mM mevalonate as indicated. After incubation at 37 °C for 5 h, the cells were harvested and subjected to cell fractionation. Aliquots of the membrane (5–32 μg of protein/lane) and nuclear extract fractions (33 μg of protein/lane) were subjected to SDS-PAGE and transferred to nylon membranes, and immunoblot analysis was carried out with 5 μg/ml monoclonal IgG-A9 (against reductase), 5 μg/ml polyclonal R139 IgG (against hamster SCAP), or 5 μg/ml monoclonal IgG-7D4 (against hamster SREBP-2). Filters were exposed to film for 1–30 s at room temperature. C, after 16 h at 37 °C, cells were switched to medium B containing 5% LPDS, 50 μM compactin, and 10 μM MG-132 in the absence (−) or presence (+) of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) plus 10 mM mevalonate as indicated. After 2 h, the cells were harvested, lysed, and subjected sequentially to immunoprecipitation and immunoblot analysis as in Fig. 1. Filters were exposed to film for 10 s at room temperature.

25-hydroxycholesterol indicates that in these cells reductase is refractory to accelerated degradation. We tested this hypothesis directly by comparing the effects of sterols and SR-12813 on reductase degradation and SREBP-2 processing in CHO-7 and SRD-14 cells (Fig. 3B and C). As shown in Fig. 3B, sterols and SR-12813 promoted the rapid degradation of reductase in parental CHO-7 cells (top panel, lanes 2 and 3), whereas the disappearance of nuclear SREBP-2 was only observed upon sterol treatment (bottom panel, lane 2). However, both SR-12813 and sterols failed to promote degradation of reductase, and sterols did not suppress nuclear SREBP-2 in the mutant SRD-14 cells (Fig. 3B, top and bottom panels, lanes 4–6). SCAP appeared to be produced at approximately equivalent levels in the CHO-7 and SRD-14 cells (Fig. 3B, 2nd panel, lanes 1–6). Consistent with their inability to promote reductase degradation in the mutant cells, sterols also failed to promote ubiquitination of reductase to appreciable levels (Fig. 3C, compare lanes 1 and 2 with 3 and 4). Thus, the inability of SRD-14 cells to carry out reductase ubiquitination and degradation explains their resistance to the growth inhibitory effects of SR-12813.

The failure of sterols to promote reductase degradation and inhibit processing of SREBP-2 indicated that SRD-14 cells are defective in a common component required for sterol regulation of both SCAP and reductase. We reasoned that this defective component was most likely one of the Insig proteins, considering their participation in sterol regulation of both proteins. Hence, membranes from CHO-7 and SRD-14 cells were isolated and subjected to immunoblot analysis with polyclonal anti-Insig-1 antibodies (Fig. 4A). To ensure maximal expression of Insig-1, which is a target gene of SREBP-2 (1, 2), the cells were incubated in sterol-depleting medium for 16 h and treated for an additional 5 h in the absence or presence of sterols prior to harvesting. Some of the dishes of cells also received MG-132 to inhibit the activity of proteasomes. In the absence of MG-132, Insig-1 was barely detectable in the anti-Insig-1 immunoblots, regardless of the absence or presence of sterols (Fig. 4A, lanes 1 and 2). However, treatment of the cells with MG-132 led to the appearance of two bands at 28 and 25 kDa (Fig. 4A, lanes 3 and 4), which likely results from the use of two start sites for translation, as has been reported for the human Insig-1 protein (1). The stabilization of Insig-1 by MG-132 indicates that the protein is rapidly degraded by proteasomes. In contrast to those from CHO-7 cells, membranes from the SRD-14 cells lacked detectable Insig-1 when incubated in the absence or presence of sterols and/or MG-132 (Fig. 4A, lanes 5–8).

We next conducted a series of experiments designed to assess
the nature of the genetic defect that leads to the absence of Insig-1 protein in SRD-14 cells. Fig. 4A shows a Northern blot, comparing the amounts of Insig-1 mRNA in CHO-7 and SRD-14 cells. Insig-1 mRNA was present in the parental cells when they were incubated in the absence of sterols (Fig. 4B, lane 1), and it declined when the cells were treated with sterols (lane 2), a result of the sterol-dependent suppression of nuclear SREBP-2. In contrast, Insig-1 mRNA failed to be detected in SRD-14 cells, regardless of the absence or presence of sterols (Fig. 4B, lanes 3 and 4). To determine whether the absence of Insig-1 mRNA in SRD-14 cells is due to a gross chromosomal deletion or rearrangement of the INSIG-1 gene, we performed Southern blots with genomic DNA isolated from CHO-7 and SRD-14 cells (Fig. 4C). EcoRI and KpnI digestions produced hybridizable fragments that had an identical size for CHO-7 and SRD-14 cells (Fig. 4C, lanes 1 and 4). In contrast, the BamHI-digested SRD-14 cell DNA failed to produce the 8- and 5-kb hybridizable bands present in the corresponding digest of DNA from CHO-7 cells (Fig. 4C, lanes 5 and 6). These results indicate that the absence of detectable Insig-1 mRNA and protein in SRD-14 cells is caused by the a partial deletion of the INSIG-1 gene.

In mutant cell lines identified previously, the persistence of nuclear SREBP-2 upon 25-hydroxycholesterol treatment was indicative of the ability of the cells to survive chronic treatment with the oxysterol (9, 12, 27–29). However, in the experiment of Fig. 3 we observed that despite their resistance to sterol-mediated suppression of nuclear SREBP-2 (Fig. 3B), SRD-14 cells were only partially resistant to growth in 25-hydroxycholesterol (Fig. 3A). Considering that, in Fig. 3B, the extent to which sterols influenced SREBP-2 processing was assessed after treating cells for 5 h, we designed an experiment to compare the kinetics of sterol-dependent suppression of nuclear SREBP-2 (Fig. 3C). To determine the amount of mRNAs encoding SREBP-2, HMG-CoA synthase, HMG-CoA reductase, and the LDL receptor, by quantitative real time PCR analysis; and the results are presented graphically in Fig. 5, B–F. The mRNAs encoding SREBP-2 target genes in CHO-7 cells declined with sterol treatment at rates that mirrored those of nuclear SREBP-2 suppression, and again, we found the sterol response of the SRD-14 cells was
markedly delayed. As expected, Insig-1 mRNA was not detected in the mutant cells and exhibited a similar response to sterols as the other SREBP-2 target genes in wild type cells (Fig. 5B). Insig-2 mRNA was present at similar levels in CHO-7 and SRD-14 cells and remained constant throughout the time course in both cell lines (Fig. 5C).

To exclude the possibility that the rapid decline of nuclear SREBP-2 in wild type cells resulted from the use of high levels (1 μg/ml) of 25-hydroxycholesterol in the experiments of Fig. 5, we next performed a 25-hydroxycholesterol dose-curve experiment at 5 and 16 h (Fig. 6). In the nuclei of CHO-7 cells, 25-hydroxycholesterol evoked a dose-dependent decrease in processed SREBP-2 after 5 h of treatment (Fig. 6, bottom panel, lanes 1–4), and this sterol effect was more pronounced after 16-h treatments (bottom panel, compare lanes 1–4 with lanes 5–7). A similar response was observed for mRNAs encoding each SREBP-2 target gene (Fig. 6, A–H). A minimal reduction of nuclear SREBP-2 was found in SRD-14 cells treated with 0.3 μg/ml 25-hydroxycholesterol at the 5-h time point (Fig. 6, lane 11). However, treatment of the mutant cells for 16 h with 0.1 μg/ml 25-hydroxycholesterol led to partial suppression of SREBP-2 processing (Fig. 6, lane 13), and complete suppression was observed in those cells treated with 0.3 μg/ml of the sterol (lane 14). After 5 h, target gene expression in the SRD-14 cells was somewhat more resistant to 25-hydroxycholesterol than were their parental CHO-7 cells (Fig. 6, A, C, D, E, and G), yet following 16 h of treatment, target gene expression in both cell lines responded similarly to the oxysterol (Fig. 6, B, D, F, and H). Considered together, the results of Figs. 5 and 6 indicate that Insig-1 mediates the rapid suppression of nuclear SREBP-2 promoted by 25-hydroxycholesterol, whereas another component of the sterol-regulatory system, likely Insig-2, orchestrates the more gradual sterol response that can be observed between 8 and 16 h in the absence of Insig-1.

We sought next to determine whether overexpression of Insig-1 or Insig-2 would restore sterol regulation of reductase ubiquitination/degradation and SREBP-2 processing after 5 h. SRD-14 cells were transfected with pCMV-Insig-1-Myc and pCMV-Insig-2-Myc, expression plasmids encoding full-length human Insig-1 and Insig-2 followed by six tandem copies of the c-Myc epitope, and clones that expressed equivalent levels of Insig-1 or Insig-2 would restore sterol regulation of reductase and suppression of nuclear SREBP-2 promoted by 25-hydroxycholesterol, whereas another component of the sterol-regulatory system, likely Insig-2, orchestrates the more gradual sterol response that can be observed between 8 and 16 h in the absence of Insig-1. We sought next to determine whether overexpression of Insig-1 or Insig-2 would restore sterol regulation of reductase ubiquitination/degradation and SREBP-2 processing after 5 h. SRD-14 cells were transfected with pCMV-Insig-1-Myc and pCMV-Insig-2-Myc, expression plasmids encoding full-length human Insig-1 and Insig-2 followed by six tandem copies of the c-Myc epitope, and clones that expressed equivalent levels of the Insig proteins were isolated (Fig. 7, bottom panel, lanes 7–12). Fig. 7A shows that in CHO-7 cells, sterols and SR-12813 promoted complete degradation of reductase (top panel, lanes 2 and 3), and sterols fully suppressed nuclear SREBP-2 (3rd panel, lane 2). The SRD-14 cells, as expected, were resistant to both reagents (Fig. 7A, top 3rd panel, lanes 4–6). In the SRD-14 cells stably overexpressing Insig-1-Myc or Insig-2-Myc, regulated degradation of reductase and suppression of nuclear SREBP-2 was completely restored (Fig. 7A, top three panels, lanes 7–12). Overexpression of Insig-1-Myc or Insig-2-Myc in the mutant cells also restored the sensitivity of SREBP-2 processing to low levels of 25-hydroxycholesterol (Fig. 7B). Finally, regulated ubiquitination of reductase was fully restored in the SRD-14 cells upon overexpression of Insig-1 or Insig-2 (Fig. 7C). Considered together, these results demonstrate that the
regulatory defects exhibited by SRD-14 cells can be completely restored upon stable transfection of either Insig-1-Myc or Insig-2-Myc.

DISCUSSION

The data presented here describe the isolation and characterization of a new line of mutant CHO cells, designated SRD-14, resistant to chronic selection with the 1,1-bisphosphonate ester SR-12813. We chose to select mutagenized CHO-7 cells with SR-12813 for several reasons. 1) SR-12813 replaced sterols in promoting Insig-dependent degradation and ubiquitination of reductase but not ER retention of the SCAP/H\textsubscript{18528}SREBP-2 complex (Fig. 1). 2) CHO-7 cells failed to grow in the presence of SR-12813, and this was overcome by the addition of exogenous cholesterol to the growth medium (Fig. 2A). 3) SR-12813 did not influence the stability of degradation-resistant mutant forms of reductase (Fig. 1D), and consequently, the drug failed to kill cells that expressed the mutant but not the wild type form of full-length reductase (Fig. 2B). The mutant cells that survived SR-12813 selection did so because they failed to accelerate degradation of reductase (Fig. 3, B and C). This failure was due to the absence of Insig-1 mRNA and protein that resulted from a partial deletion of the INSIG-1 gene (Fig. 4). SRD-14 and 7 other lines of Insig-1-deficient cells were produced from a single round of mutagenesis of 2.5 \times 10^7 CHO-7 cells, followed by selection in SR-12813. The repeated isolation of Insig-1-deficient cells in our experiments indicates that CHO-7 cells may only have one functional copy of the INSIG-1 gene, and thus a single mutagenic event can destroy the single copy of the INSIG-1 gene, leading to Insig-1 deficiency. The failure of SRD-14 cells to degrade reductase in response to SR-12813 or sterols appears to result from their deficiency in total Insig. This is indicated by the restoration of regulated ubiquitination and degradation of reductase upon overexpression of either Insig-1-Myc or Insig-2-Myc in the SRD-14 cells (Fig. 7).
**Fig. 7.** Stable transfection of SRD-14 cells with pCMV-Insig-1-Myc or pCMV-Insig-2-Myc restores regulation of HMG-CoA reductase degradation/ubiquitination and SREBP-2 processing mediated by sterols and SR-12813. CHO-7, SRD-14, SRD-14/Insig-1, and SRD-14/Insig-2 cells were set up on day 0 at 5 x 10^5 cells per 100-mm dish in medium B containing 5% LPDS. On day 2, the cells were switched to medium B supplemented with 5% LPDS, 10 μM compactin, and 50 μM mevalonate. A and C, after 16 h at 37°C, the cells were switched to medium B containing 5% LPDS and 50 μM compactin in the absence (−) or presence (+) of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) or 10 μM SR-12813 plus 10 mM mevalonate, as indicated. B, the cells were switched to medium B containing 5% LPDS, 50 μM compactin, and the indicated concentrations of 25-hydroxycholesterol. A and B, following incubation at 37°C for 5 h, the cells were harvested and subjected to cell fractionation, and aliquots of the membrane (4–50 μg of protein/lane) and nuclear extract (11–27 μg of protein/lane) fractions were subjected to SDS-PAGE. Immunoblot analysis was carried out with 5 μg/ml monoclonal IgG-A9 (against reductase), 5 μg/ml monoclonal IgG-7D4 (against hamster SREBP-2), and 1 μg/ml anti-Myc monoclonal IgG (against Insig-1 and Insig-2). Filters were exposed to film for 1–30 s at room temperature. C, after incubation for 2 h at 37°C, cells were harvested, lysed, and sequentially subjected to immunoprecipitation, SDS-PAGE, and immunoblot analysis as described in the legend to Fig. 3. Filters were exposed to film for 10 s at room temperature.

**A. Wild Type Cells (CHO-7)**

**B. Insig-1 Cells (SRD-14)**

**Fig. 8.** Model for Insig-mediated regulation of SREBP processing and HMG-CoA reductase degradation. A, in sterol-depleted wild type CHO-7 cells, Insig-1 and Insig-2 dissociate from the SCAP-SREBP complex, allowing its entry into budding vesicles and translocation to the Golgi where SREBPs are processed. Meanwhile, reductase is stable and produces mevalonate. In the nucleus, processed SREBPs activate transcription of genes encoding cholesterol biosynthetic enzymes and Insig-1. The resulting rise of Insig-1 protein and intracellular accumulation of sterols eventually leads to the rapid suppression of SREBP processing through the sterol-dependent binding of Insig-1 to SCAP in the ER, preventing it from escorting SREBPs to the Golgi for proteolytic activation. At the same time Insig-1 mediates sterol-accelerated degradation of reductase. In the absence of SREBP processing, Insig-1 expression decreases, yet SREBP processing continues to be suppressed because the constitutively low level of Insig-2 is enough to retain the SCAP-SREBP complex in the ER. B, in the Insig-1-deficient SRD-14 cells, sterol-dependent suppression of SREBP processing occurs at a slower rate than in wild type cells, because of the low abundance of Insig-2. However, Insig-2 alone is not sufficient to accelerate reductase degradation, and this renders SRD-14 cells resistant to SR-12813.
The growth of SRD-14 cells in SR-12813 indicates that Insig-2 alone cannot carry out reductase degradation, whereas their inability to grow in the presence of 25-hydroxycholesterol suggests that regulation of SREBP processing must be intact upon prolonged sterol treatment (Fig. 3A). Thus, we compared the kinetics of sterol-dependent suppression of SREBP-2 processing in parental CHO-7 and mutant SRD-14 cells (Figs. 5 and 6). In the parental cells, sterols rapidly suppressed SREBP-2 processing and mRNAs encoding Insig-1, and other SREBP-2 target genes fell accordingly. In the absence of sterols, we approximate that Insig-1 expression exceeds that of Insig-2 by 10-fold in CHO cells, thus 90% of total Insig is susceptible to sterol-dependent suppression. As expected, Insig-2 alone mediated sterol suppression of SREBP-2 processing in SRD-14 cells, although at a slower rate than wild type cells. Thus, it appears that cells require their total complement of Insigs to carry out sterol-accelerated degradation of reductase, whereas sterol suppression of SREBP processing can occur at lower Insig levels. These results are consistent with those we obtained with RNAi experiments, where transient knockdown of Insig-1 and Insig-2 blocked sterol-accelerated degradation of reductase, but sterol-mediated suppression of SREBP processing remained intact (data not shown). This disparity may reflect differences in the affinities of reductase and SCAP for sterols and/or Insigs. Finally, stable overexpression of Insig-1 or Insig-2 in SRD-14 cells restored the rapid suppression of SREBP processing (Fig. 7), thus reflecting the redundant role of the two proteins in mediating sterol regulation of SREBP as well as reductase.

Taken together, the current results provide compelling genetic evidence for the important role Insig-1 plays in mediating the differential actions of Insig-1 and Insig-2 in regulating sterol-mediated suppression of SREBP processing as long as intracellular sterols remain high. Considering that under conditions of sterol deprivation Insig-1 accounts for 90% of total Insigs, the ability of SREBP-2 to modulate levels of its inhibitor provides a reset mechanism that permits cells to rapidly modulate SREBP processing according to their demands for sterols. When cellular sterols are high, Insig-2 is the only form of Insig present, and thus the cells can respond rapidly to cholesterol depletion. On the other hand, when SREBPs are actively processed, the INSIG-1 gene is activated, and the increase in total Insig primes the cells for rapid suppression when sterol levels rise.

In conclusion, the analysis of regulated SREBP processing and accelerated degradation of reductase in the Insig-1-deficient SRD-14 cells has revealed an interesting circuitry underlying sterol regulation; however, the complete resolution of this unique regulatory system requires the availability of Insig-2-deficient cells, as well as cells lacking both Insig-1 and Insig-2.

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