Insig-1 and Insig-2, a pair of endoplasmic reticulum (ER) membrane proteins, mediate feedback control of cholesterol synthesis through their sterol-dependent binding to the following two polytopic ER membrane proteins: sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Sterol-induced binding of Insigs to SCAP prevents the proteolytic processing of SREBPs, membrane-bound transcription factors that enhance the synthesis of cholesterol, by retaining complexes between SCAP and SREBP in the ER. Sterol-induced binding of Insigs to SCAP prevents their ubiquitination and ER-associated degradation of the enzyme, thereby slowing a rate-controlling step in cholesterol synthesis. Here we report the isolation of a new line of mutant Chinese hamster ovary cells, designated SRD-15, deficient in both Insig-1 and Insig-2. The SRD-15 cells were produced by γ-irradiation of Insig-1-deficient SRD-14 cells, followed by selection in high levels of the oxysterol, 25-hydroxycholesterol. Sterols neither inhibit SREBP processing nor promote reductase ubiquitination/degradation in SRD-15 cells. Sterol regulation of SREBP processing and reductase ubiquitination/degradation is fully restored in SRD-15 cells when they are transfected with expression plasmids encoding either Insig-1 or Insig-2. These results demonstrate an absolute requirement for Insig proteins in the regulatory system that mediates lipid homeostasis in animal cells.

It has become increasingly evident that Insig-1 and Insig-2, a pair of related endoplasmic reticulum (ER)-localized membrane proteins, are crucial for homeostatic control of cholesterol synthesis in animal cells. Insigs coordinate the synthesis of cholesterol and other lipids through their sterol-dependent binding to the following two polytopic ER membrane proteins: SREBP cleavage-activating protein (SCAP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). SCAP controls the activation of SREBPs, a family of membrane-bound transcription factors that activate transcription of all the known genes encoding cholesterol biosynthetic enzymes (1). Excess sterols promote the binding of SCAP to Insigs, a reaction that leads to the ER retention of SCAP and prevents delivery of its bound SREBPs to the Golgi for proteolytic release from membranes (2, 3). In the absence of SREBP activation, levels of the mRNAs encoding SREBP target genes fall, and cholesterol synthesis is suppressed. The sterol-induced binding of reductase to Insigs leads to its ubiquitination and subsequent degradation. This reaction is a part of a complex, multivalent feedback mechanism that governs the levels and activity of reductase (4). The reductase-catalyzed conversion of HMG-CoA to mevalonate is a rate-determining step in the synthesis of cholesterol and nonsterol isoprenoids (5). Accelerated degradation of reductase slows the rate at which mevalonate is produced, which in turn halts the synthesis of cholesterol. Together, these Insig-mediated reactions (ER retention of SCAP and accelerated degradation of reductase) ensure that cells maintain the production of important nonsterol by-products of mevalonate metabolism, while avoiding the overaccumulation of potentially toxic end products such as cholesterol.

When SCAP and reductase are overexpressed in Chinese hamster ovary (CHO) cells by transfection, neither protein is subjected to sterol regulation (2, 6). Overexpression of Insig-1 or Insig-2 restores sterol-mediated ER retention of overexpressed SCAP and sterol-accelerated degradation of overexpressed reductase. The sterol-dependent binding of SCAP and reductase to Insigs occurs through a region of both proteins that consists of five of their eight membrane-spanning helices (2, 6–9). The amino acid sequence of the Insig-binding sites in SCAP and reductase bears significant identities to each other, and the region has become known as the sterol-sensing domain (10, 11). Point mutations within the sterol-sensing domains of SCAP and reductase prevent their binding to Insigs, thereby abolishing sterol-mediated ER retention of mutant SCAP-SREBP complexes and sterol-dependent ubiquitination/degradation of mutant reductase (2, 9, 12).

The two human Insig proteins are 59% identical and are predicted to share a similar topology with both proteins containing six membrane-spanning helices (13). Despite these similarities, Insig-1 and Insig-2 differ in their mode of regulation (3, 14). In cultured cells, the INSIG-1 gene is a target of SREBPs, and the transcription rate of the gene parallels the nuclear content of processed SREBPs. Transcription of the INSIG-2 gene is controlled by two promoters that give rise to alternative mRNA 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 does not vary in cultured cells; it is not influenced by SREBPs. The Insig-2a transcript is exclusively expressed in the liver and is down-regulated by insulin.

We recently characterized a line of mutant CHO cells, designated SRD-14, in which Insig-1 mRNA and protein are not produced as a result of a partial deletion of one copy of the INSIG-1 gene and a loss of function of the other copy (15).
SRD-14 cells were produced by γ-irradiation of CHO cells, followed by selection in medium supplemented with SR-12813. SRD-12813 belongs to a class of cholesterol-lowering 1,1-bisphosphonate esters that mimic sterols in accelerating reductase degradation but do not block SREBP processing. As a result of their Insig-1 deficiency, SRD-14 cells did not respond to SR-12813 by promoting reductase ubiquitination and degradation. The cells also had a partial resistance to the regulatory sterol, 25-hydroxycholesterol. In the SRD-14 cells, 25-hydroxycholesterol absolutely failed to promote reductase ubiquitination and degradation. However, 25-hydroxycholesterol did block SREBP processing, although this effect required a longer exposure to the oxysterol in SRD-14 cells as compared with wild-type cells. Resistance to SR-12813 and 25-hydroxycholesterol was completely corrected by the overexpression of either Insig-1 or Insig-2. Considering that in wild-type CHO cells, Insig-1 accounts for −90% of total Insigs (15), we proposed that the remaining Insig-2 was sufficient to maintain partial sterol regulation of SCAP-SREBP but not reductase. Definitive proof of this hypothesis requires an analysis of the SCAP-SREBP pathway in cells lacking both Insig-1 and Insig-2.

In the current studies, we describe the generation, isolation, and characterization of mutagenized SRD-14 cells that are totally resistant to 25-hydroxycholesterol. This cell line, designated SRD-15, was found to harbor a deletion of one allele of the INSIG-2 gene, and Insig-2 mRNA expression was reduced to less than 20% of wild-type cells. As a result of their combined deficiencies in Insig-1 (100% deficiency) and Insig-2 (80% deficiency), SRD-15 cells were completely refractory to sterol-mediated actions on SREBP processing, as well as reductase degradation. The experiments with the SRD-15 cells provide genetic proof that cells require at least one Insig protein in order to exhibit normal feedback regulation of cholesterol metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials—**We obtained sterols (25-hydroxycholesterol and cholesterol) from Steraloids, Inc. (Newport, RI); MG-132 was from Calbiochem; methyl- and hydroxypropyl-β-cyclodextrin were from Cycloextrin Technologies Development, Inc.; DECAtemplate β-actin mouse was from Ambion; horseradish peroxidase-conjugated, donkey anti-mouse, and anti-rabbit IgGs were from Jackson ImmunoResearch. Lipoprotein-deficient serum (d > 1.215 g/ml) was prepared from newborn calf serum by ultracentrifugation (16). Solutions of sodium mevalonate, sodium carbonate, and glyceraldehyde-3-phosphate dehydrogenase (invariant control) were purchased from Sigma-Aldrich. IgG-2179, a rabbit polyclonal antibody (previously undescribed) prepared against bovine ubiquitin (Santa Cruz Biotechnology); and 5 μg/ml of G418, a mouse monoclonal antibody against c-Myc purified from the culture medium of hybridoma clone 9F10 (American Type Culture Collection) were purchased from Sigma-Aldrich. A supplemented medium containing 5% fetal calf serum, 5 μg/ml cholesterol, 1 mM mevalonate, and 20 μM sodium olate. On day 1, the cells were transfected with 1 μg of pCMV-Insig-1-Myc or pCMV-Insig-2-Myc, expression plasmids encoding human Insig-1 and Insig-2 followed by six tandem copies of the c-Myc epitope (2, 3), using the FuGENE 6 transfection reagent as described (23). On day 2, cells were switched to the identical medium supplemented with 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 immunoblotting with anti-Myc antibodies. Cells harboring a transgene were cloned by limiting dilution and maintained in medium A containing 5% fetal calf serum, 5 μg/ml cholesterol, 1 mM mevalonate, 20 μM sodium olate, and 500 μg/ml G418 at 37°C, 8–9% CO2.

**Cell Fractionation and Immunoblot Analysis—**The pooled cell pellets from triplicate dishes of cells were used to isolate membrane fractions and/or nuclear extract fractions as described previously (6, 9). Aliquots of membrane fractions and/or nuclear extract fractions were subjected to SDS-PAGE; the proteins were transferred to Hybond-C extra nitrocellulose filters (Millipore), and immunoblot analysis was carried out as described (6). Primary antibodies used for immunoblotting are as follows: IgG-7D4, a mouse monoclonal antibody against the NH2 terminus of hamster SREBP-2 (24); IgG-9E10, a mouse monoclonal antibody against c-Myc purified from the culture medium of hybridoma clone 9F10 (American Type Culture Collection); IgG-9A1, a mouse monoclonal antibody against the catalytic domain of hamster HMG-CoA reductase (amino acids 450–887) (25); IgG-P1D1, a mouse monoclonal antibody against bovine ubiquitin (Santa Cruz Biotechnology); and 5 μg/ml of IgG-2179, a rabbit polyclonal antibody (previously undescribed) prepared by immunizing rabbits with a bacterially produced and purified fusion protein encoding an NH2-terminal Hisa tag, followed by amino acids 1–125 of hamster SREBP-1c (26).

**Real Time PCR and Northern and Southern Blot Analysis—**The real-time PCR protocol for real time PCR was identical to that described by Liang et al. (27). Total RNA was isolated from CHO-7, SRD-14, and SRD-15 cells using the RNeasy kit (Qiagen) according to the manufacturer’s instructions and subjected to reverse transcription reactions. Triplicate samples of reverse-transcribed total RNA were subjected to real time PCR quantification using forward and reverse primers for hamster Insig-1, Insig-2, SCAP, HMG-CoA reductase, HMG-CoA synthase, LDL receptor, and glyceroldehyde-3-phosphate dehydrogenase (invariant control) (15). Relative amounts of mRNAs were calculated using the comparative C(T) method. Hamster Insig-2 and hamster SCAP cDNA probes for Northern and Southern blot analyses were prepared by PCR amplification of mouse-reversed transcribed total RNA isolated from the following forward and reverse primers: Insig-2: 5′-CCACCAACCTGCAGAGGGCCAC-3′ and 5′-CCAAAGCGCCTTGACTCGTGGAGGTTTCCG-3′; SCAP: 5′-GATGCTATTGTTGTCGGTGAAAC-3′ and 5′-GACAGCTTCTCCTCTCTTGTG-3′. The cDNA probe for hamster Insig-1 was also generated by PCR as described previously (15). The resulting PCR products and the mouse β-actin probe were labeled with [α-32P]dCTP using the Megaprime DNA Labeling System (Amersham Biosciences). In addition, the PCR products of the Insig-2 reaction were digested with KpnI, and the resulting two fragments (corresponding to nucleotides 1–216 and 217–678 of the Insig-2 DNA, respectively) were used as probes in Southern blot analyses. Total RNA and restriction enzyme-digested genomic DNA were subjected to electrophoresis and transferred to Hybond N+ membranes (Amersham Biosciences), and the filters were hybridized at 60–68°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.

**Real Time PCR Quantification and Pulse-Chase Analysis of HMG-CoA Reductase—**Cells were pulse-labeled in methionine/cysteine-free medium A, and pulse-chase analysis was carried out as described (9). Immunoprecipitation of labeled reductase from detergent lysates was carried out with polyclonal antibodies against the 60-kDa COOH-terminal domain of human reductase as described previously (9, 28). Immunoprecipitates were subjected to SDS-PAGE and transferred to Hybond C-extra nitrocellulose filters. Dried filters were exposed to an imaging plate at room temperature for 1 h, and the bands were quantified using a Phosphoimage analyzer (Amersham Biosciences).
Mutant Cells Lacking Insig-1 and Insig-2

In previous studies, we found that sterols blocked the accumulation of nuclear SREBPs in SRD-14 cells only after prolonged treatment (up to 16 h) (15). In light of this, we compared the sterol regulation of SREBP processing in wild-type CHO-7, Insig-1-deficient SRD-14, and SRD-15 cells (Fig. 2). We depleted the cells of sterols by incubating them in medium containing lipoprotein-deficient serum, the reductase inhibitor compactin (17), and the lowest level of mevalonate (50 μM) that ensures viability. Some of the cells also received various concentrations of 25-hydroxycholesterol. After 5 or 16 h, the cells were harvested and separated into membrane and nuclear extract fractions, and aliquots of the fractions were subjected to SDS-PAGE. Subsequently, immunoblot analysis of the fractions was carried out with anti-SREBP-1 (Fig. 2A, top panel) and anti-SREBP-2 (Fig. 2A, bottom panel) antibodies. In untreated cells, bands corresponding to the processed, nuclear forms of SREBP-1 and SREBP-2 were observed at the 5- and 16-h time points (Fig. 2A, top and bottom panels, lanes 1, 5, 9, 13, 17, and 21). In CHO-7 cells, 25-hydroxycholesterol caused the disappearance of both nuclear SREBP-1 and SREBP-2 in a dose-dependent manner after 5 and 16 h of treatment (Fig. 2A, top and bottom panels, lanes 2–4 and 14–16). After 5 h of treatment, 25-hydroxycholesterol did not appreciably reduce nuclear SREBP-1 and SREBP-2 levels in either SRD-14 or SRD-15 cells (Fig. 2A, top and bottom panels, lanes 6–8 and 10–12). At the 16-h time point, 25-hydroxycholesterol completely blocked processing of both SREBPs in CHO-7 and SRD-14 cells (Fig. 2A, lanes 14–16 and 18–20), whereas SREBP processing in the SRD-15 cells was fully resistant to oxysterol-mediated suppression (Fig. 2A, top and bottom panels, lanes 22–24).

In addition to inhibition by 25-hydroxycholesterol, SREBP processing is inhibited by high concentrations of cholesterol added in methyl-β-cyclodextrin (29). The experiment of Fig. 2B shows that SRD-15 cells are resistant to cholesterol as well as 25-hydroxycholesterol. When delivered to CHO-7 cells as complexes with methyl-β-cyclodextrin, 25-hydroxycholesterol at 2.5 μM and cholesterol at 25 μM inhibited accumulation of nuclear SREBP-2 (Fig. 2B, lanes 3, 4, and 7), whereas no such effect was seen in SRD-15 cells (Fig. 2B, lanes 10, 11, and 14).

We next compared the steady state levels of endogenous reductase and nuclear SREBP-2 in CHO-7 and SRD-15 cells incubated in lipoprotein-deficient serum with or without compactin supplementation for 16 h. Immunoblotting membranes with anti-reductase revealed that in the absence of compactin the steady state level of reductase was low in wild-type CHO-7 cells (Fig. 3A, top panel, lane a), and this rose with compactin treatment in a dose-dependent fashion (Fig. 3A, lanes b–d). This finding is consistent with previous observations, which demonstrated that blocking mevalonate metabolism reduces regulatory molecules that normally govern the reductase regulatory system, resulting in a compensatory increase of the enzyme in ER membranes (30). In untreated SRD-15 cells, the steady state levels of reductase were equal to that of compactin-treated CHO-7 cells (Fig. 3A, compare lanes d and e) and failed to be increased by compactin (Fig. 3A, lanes f–h). Nuclear SREBP-2 followed a similar pattern to that of reductase (Fig. 3A, bottom panel). Compactin treatment led to an increased nuclear accumulation of SREBP-2 in the wild-type cells (Fig. 3A, lanes a–d), whereas the drug had no effect on the elevated levels of nuclear SREBP-2 in SRD-15 cells (Fig. 3A, lanes e–h). In parallel experiments, we isolated total RNA from the treated cells and measured the levels of mRNAs for SCAP, reductase, HMG-CoA synthase, and the LDL receptor by quantitative real time PCR. As expected, SCAP mRNA was constant in CHO-7 and SRD-15 cells, regardless of the absence or presence of compactin. In CHO-7 cells, compactin treatment led to a dose-dependent increase in the expression reductase, HMG-CoA synthase, and LDL receptor mRNAs (31). This finding is consistent with previous observations, which demonstrated that blocking mevalonate metabolism reduces regulatory molecules that normally govern the reductase regulatory system, resulting in a compensatory increase of the enzyme in ER membranes (30). In untreated SRD-15 cells, the steady state levels of reductase were equal to that of compactin-treated CHO-7 cells (Fig. 3A, compare lanes d and e) and failed to be increased by compactin (Fig. 3A, lanes f–h). Nuclear SREBP-2 followed a similar pattern to that of reductase (Fig. 3A, bottom panel). Compactin treatment led to an increased nuclear accumulation of SREBP-2 in the wild-type cells (Fig. 3A, lanes a–d), whereas the drug had no effect on the elevated levels of nuclear SREBP-2 in SRD-15 cells (Fig. 3A, lanes e–h). In parallel experiments, we isolated total RNA from the treated cells and measured the levels of mRNAs for SCAP, reductase, HMG-CoA synthase, and the LDL receptor by quantitative real time PCR. As expected, SCAP mRNA was constant in CHO-7 and SRD-15 cells, regardless of the absence or presence of compactin. In CHO-7 cells, compactin treatment led to a dose-dependent increase in the expression reductase, HMG-CoA synthase, and LDL receptor mRNAs (31).
Proteolytic processing of SREBPs is refractory to sterol regulation in SRD-15 cells. A, CHO-7, SRD-14, and SRD-15 cells were set up on day 0 at 5 × 10⁵ cells per 100-mm dish in medium A supplemented with 5% lipoprotein-deficient serum. On day 2, the cells were refed medium A containing 5% lipoprotein-deficient serum, 10 µM sodium compactin, and 50 µM sodium mevalonate. Some of the cells received the same medium supplemented with the indicated concentration of 25-hydroxycholesterol. Sterols were added to the cells in a staggered fashion, such that all of the cells could be harvested together after incubations for 5 or 16 h at 37 °C. After the incubations, the cells were harvested and subjected to cell fractionation as described under "Experimental Procedures." Aliquots of the membrane (40 µg of protein/lane) and nuclear extract (32 µg of protein/lane) fractions were subjected to SDS-PAGE and transferred to nylon membranes, and immunoblot analysis was carried out with 5 µg/ml IgG-2179 (against hamster SREBP-1) or 5 µg/ml IgG-1219 (against hamster SREBP-2). Filters were exposed to film for 5–20 s at room temperature. B, CHO-7 and SRD-15 cells were set up and refed as in A. On day 3, the cells were incubated for 1 h in medium A supplemented with 5% lipoprotein-deficient serum, 50 µM compactin, 50 µM mevalonate, and 1% (w/v) hydroxypropyl-β-cyclodextrin. Subsequently, the cells were washed with phosphate-buffered saline and subjected to an additional 6 h incubation at 37 °C in medium A containing 5% lipoprotein-deficient serum, 50 µM compactin, 50 µM mevalonate, and the indicated concentration of 25-hydroxycholesterol (lanes 1–4 and 8–11) or cholesterol (lanes 5–7 and 12–14) complexed with methyl-β-cyclodextrin. Following the incubation, the cells were harvested and subjected to cell fractionation and immunoblot analysis as in A. Filters were exposed to film at room temperature for 30 s.

The failure of SRD-15 cells to respond to 25-hydroxycholesterol and cholesterol suggested that these cells were deficient in Insig-2 as well as Insig-1. To test this hypothesis, we pre-treated CHO-7, SRD-14, and SRD-15 cells in the absence and presence of sterols for 16 h (Fig. 4A) and isolated total RNA. Northern blotting with a radiolabeled Insig-1 cDNA probe revealed the presence of Insig-1 mRNA in untreated CHO-7 cells (Fig. 4A, top panel, lane 1) which declined when the cells were treated with sterols (Fig. 4A, lane 2), a result of the sterol-dependent reduction in nuclear SREBPs. As expected, Insig-1 was not detected in SRD-14 and SRD-15 cells (Fig. 4A, lanes 3–6). CHO-7 and SRD-14 cells expressed approximately equivalent levels of Insig-2 mRNA, regardless of sterol treatment (Fig. 4A, middle panel, lanes 1–4). However, the level of Insig-2 mRNA in SRD-15 cells was markedly reduced in comparison to that in the parental SRD-14 and wild-type CHO-7 cells (Fig. 4A, lanes 5 and 6). To quantify the amount of Insig-2 mRNA remaining in SRD-15 cells, we subjected total RNA to reverse transcription reactions and quantitative real time PCR analysis (Fig. 4B). Consistent with the observation in Fig. 4A, Insig-1 mRNA was only detected in wild-type CHO-7 cells, and its level was suppressed by sterols. The mRNAs encoding reductase, HMG-CoA synthase, and LDL receptor were similarly suppressed by sterols in CHO-7 and SRD-14 cells, but not in the SRD-15 cells. Insig-2 mRNA was present in the CHO-7 and SRD-14 cells at approximately equivalent levels. However, in the SRD-15 cells, Insig-2 mRNA was reduced to less than 20% of that in CHO-7 and SRD-14 cells. The Insig-2 mRNA that remained in the SRD-15 cells had a normal sequence as shown by cDNA cloning and sequencing. We also observed an 80% decrease of Insig-2 mRNA in 18 independent isolates from the mutagenesis experiment that yielded SRD-15 cells. Moreover, this phenotype of the SRD-15 cells has been determined to be stable over a 6-month period.

---

### Table A

<table>
<thead>
<tr>
<th>CHO-7</th>
<th>SRD-14</th>
<th>SRD-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-Hydroxycholesterol (µM)</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>25-Hydroxycholesterol (µM)</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>25-Hydroxycholesterol (µM)</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th>CHO-7</th>
<th>SRD-14</th>
<th>SRD-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (µM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol (µM)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol (µM)</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

---

**Synthase, and LDL receptor mRNAs.** These mRNAs were somewhat elevated to varying degrees in SRD-15 cells, but compactin had no effect on their expression.

In the experiment of Fig. 3B, we compared the rate of reductase synthesis in CHO-7 and SRD-15 cells. Following incubation for 16 h in the absence or presence of compactin, the cells were pulse-labeled with a mixture of [³⁵S]methionine plus cysteine for increasing periods of time, after which they were harvested, lysed in detergent-containing buffer, and immunoprecipitated with polyclonal antibodies against reductase. CHO-7 (Fig. 3B, lanes a–e and k–o) and SRD-15 (Fig. 3B, lanes j–p and q–t) cells incorporated [³⁵S]radioactivity into reductase protein in a similar manner that increased with time. Notably, quantification of the gels revealed that the rate of reductase synthesis was marginally higher in the SRD-15 cells than in CHO-7 cells.

The experiment of Fig. 3C was designed to compare the rate of sterol-regulated turnover of reductase in CHO-7 and SRD-15 cells. The cells were depleted of sterols for 16 h by incubating them in lipoprotein-deficient serum containing compactin. Subsequently, the cells were pulse-labeled for 30 min with [³⁵S]methionine, after which they were washed and switched to medium containing an excess of unlabeled methionine and cysteine in the absence of sterols plus high levels (10 mM) of mevalonate (to augment sterol-accelerated degradation of the reductase (9)). In the absence of sterols, the amount of [³⁵S]labeled reductase in CHO-7 cells declined during the 6-h chase period (Fig. 3C, lanes b, d, and f), and this decline was accelerated by the addition of sterols plus mevalonate to the chase medium (Fig. 3C, lanes c, e, and g). In contrast, accelerated degradation of reductase was refractory to sterol regulation in SRD-15 cells, as indicated by the identical rate of decline in radiolabeled reductase in the absence or presence of sterols plus mevalonate (Fig. 3C, lanes i–n).
The finding of some normal Insig-2 mRNA in SRD-15 cells indicates that at least one copy of the gene is intact. To determine whether the other copy of the Insig-2 gene is deleted or rearranged, we subjected genomic DNA isolated from SRD-14 and SRD-15 cells to Southern blot analysis (Fig. 4C). Restriction enzyme-digested DNA from SRD-14 and SRD-15 cells was hybridized with four radiolabeled probes corresponding to the following: 1) the entire open reading frame of Insig-2 (nucleotides 1–678, Fig. 4C, lanes 1–6); 2) nucleotides 1–216 of the Insig-2 cDNA (Fig. 4C, lanes 7–12); 3) nucleotides 217–678 of the Insig-2 cDNA (Fig. 4C, lanes 13–18); and 4) nucleotides 1–697 of the SCAP cDNA (Fig. 4C, lanes 19–24) as a loading control. All of the bands observed in SRD-14 cells were also observed in SRD-15 cells, and no novel bands appeared in the SRD-15 digests (Fig. 4C, lanes 1–18). However, the intensity of several bands was reduced in the SRD-15 digests in comparison to quantitative real time PCR analysis using primers specific for the indicated gene. Each value represents the amount of the indicated mRNA relative to that of SCAP in CHO-7 cells incubated in the absence of compactin. B and C, CHO-7 and SRD-15 cells were set up on day 0 at 4 × 10^5 cells per 60-mm dish in medium A supplemented with 5% lipoprotein-deficient serum. On day 2, the cells were switched to medium A containing 5% lipoprotein-deficient serum with or without 10 μM compactin, 50 μM mevalonate, and 0.5 mM unlabeled methionine, and 1 mM unlabeled cysteine in the absence or presence of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) plus 10 mM mevalonate. After the indicated time of chases, the cells were harvested, lysed, and subjected to immunoprecipitation with polyclonal anti-reductase antibodies as described under “Experimental Procedures.” Aliquots of immunoprecipitates (normalized for equal protein/lane) were subjected to SDS-PAGE and transferred to nylon membranes. Filters were exposed for 12 h to an imaging plate at room temperature and scanned in a Storm 820 PhosphorImager (Amersham Biosciences), and the image was photographed.
deletion. The other copy is presumably intact, but its transcription is somehow compromised. The net result is an 80% reduction in Insig-2 mRNA.

We sought next to determine whether overexpression of Insig-1 or Insig-2 would restore sterol regulation of SREBP-2 processing after 5- and 16-h treatments (Fig. 5). SRD-15 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. We isolated clones that expressed equivalent levels of the Insig proteins (Fig. 5, bottom panel, lanes 1–12). As expected, processing of SREBP-2 in the SRD-15 cells was fully resistant to sterols after 5- or 16-h treatments (Fig. 5, top panel, lanes 1–2). Overexpression of Insig-1 or Insig-2 in SRD-15 cells restored a full response to sterols at both time points (Fig. 5, lanes 3–6).

We next determined whether sterol-dependent ubiquitination and degradation of reductase was restored by the overexpression of Insig-1 or Insig-2 in SRD-15 cells (Fig. 6). In the experiment of Fig. 6A, sterol-depleted cells were incubated for 5 h with sterols and 10 mM mevalonate, after which the cells were harvested and subjected to fractionation, and aliquots of
Myc or pCMV-Insig-2-Myc restores regulation of SREBP-2 processing mediated by sterols. Cells were set up on day 0 at 5 × 10^5 per 100-mm dish in medium A supplemented with 5% lipoprotein-deficient serum. On day 2, the cells were switched to medium A containing 5% lipoprotein-deficient serum, 10 μM compactin, and 50 μM mevalonate. Some of the cells received the same medium supplemented with a mixture of 25-hydroxycholesterol (2.5 μM) and cholesterol (25 μM) as indicated. Sterols were added to the cells in a staggered fashion, such that all of the cells could be harvested together after incubations for 5 or 16 h at 37 °C. Following the incubations, the cells were harvested and subjected to cell fractionation, and aliquots of the membrane (40 μg protein/lane) and nuclear extract (32 μg protein/lane) fractions were subjected to SDS-PAGE. Immunoblot analysis was carried out with 5 μg/ml 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 2 min (upper panel) and 5 s (lower panel) at room temperature.

The membrane fractions were immunoblotted with anti-reductase. In the SRD-15 cells, reductase was refractory to sterol-accelerated degradation (Fig. 6A, top panel, lanes 1 and 2). Overexpression of either Insig-1-Myc or Insig-2-Myc restored rapid degradation of the enzyme (Fig. 6A, lanes 3–6). To assay reductase ubiquitination, sterol-depleted cells were treated for 1 h with sterols and the proteasome inhibitor MG-132 to prevent degradation of ubiquitinated reductase. The cells were harvested, lysed, and subjected to immunoprecipitation with polyclonal anti-reductase antibodies. After electrophoresis of the immunoprecipitates, the membranes were blotted with antibodies against reductase or ubiquitin (Fig. 6B). Sterols stimulated ubiquitination of reductase in CHO-7 cells as indicated by the appearance of high molecular weight smears in the anti-ubiquitin immunoblots of the reductase immunoprecipitates (Fig. 6B, top panel, compare lanes 1 and 2). As expected, reductase ubiquitination was not stimulated by sterols in the SRD-14 or SRD-15 cells (Fig. 6B, lanes 3–6). Ubiquitination of reductase was restored in the SRD-15 cells when they overexpressed either Insig-1 or Insig-2 (Fig. 6B, lanes 7–10).

**Discussion**

The data presented here provide compelling genetic evidence for a requirement of both Insigs in the normal regulation of cholesterol metabolism in cultured cells. This conclusion was drawn from the characterization of a new line of mutant CHO cells, designated SRD-15, with genetic deficiencies in both Insig-1 and Insig-2. SRD-15 cells were produced by mutagenesis of Insig-1-deficient SRD-14 cells, followed by selection for growth in the presence of 25-hydroxycholesterol. It was determined previously that SREBP processing in SRD-14 cells remained sensitive to prolonged treatment with 25-hydroxycholesterol, despite their Insig-1 deficiency. As a result, SRD-14 cells will not grow indefinitely in the presence of 25-hydroxycholesterol. We reasoned that this residual sterol regulation was attributable to the remaining Insig-2 in SRD-14 cells, and this hypothesis was substantiated in the current studies by two observations. First, SRD-15 cells grow in concentrations of 25-hydroxycholesterol that kill SRD-14 cells (Fig. 1). Second, SREBP processing in the SRD-15 cells is completely refractory to sterol regulation, even after prolonged treatments (Fig. 2), and elevated in SRD-15 in comparison to CHO-7 cells (Fig. 3). Moreover, the steady state levels of reductase were also increased in the mutant SRD-15 cells compared with their wild-type counterparts (Fig. 3). The sterol regulation of SREBP processing, as well as sterol-induced ubiquitination and degradation of reductase, was restored in SRD-15 cells by the overexpression of either Insig-1 or Insig-2 (Figs. 5 and 6). This indicates that the sterol-resistant phenotype of SRD-15 cells results from their deficiency in the amount of total Insig. This conclusion is supported by our failure to find mutations in any of the other genes known to participate in the SREBP pathway (SREBP-1, SREBP-2, and SCAP) as determined by cDNA cloning and sequencing (data not shown).

Considered together with previous observations in SRD-14 cells (15), the current data have implications regarding the role of Insigs in regulating the processing of SREBPs and the degradation of reductase. First, it seems that the initial response of cells to sterols requires high levels of total Insig and is
Mutant Cells Lacking Insig-1 and Insig-2

25249

categorized by the rapid suppression of SREBP processing and the stimulation of reductase degradation. This follows from the observation that within 5 h of addition to sterol-depleted cells, sterols neither suppressed SREBP processing nor accelerated reductase degradation in Insig-1-deficient SRD-14 cells, even though the cells possess normal levels of Insig-2. This indicates that in wild-type cells, these rapid sterol-mediated reactions are primarily mediated by Insig-1, which we estimate accounts for 90% of total Insig. Moreover, the rapid response can be restored by the overexpression of either Insig-1 or Insig-2 in the SRD-14 cells.

Another implication relates to the delay in sterol-mediated inhibition of SREBP processing in SRD-14 cells (Fig. 2). This delay may indicate that the SCAP-SREBP complex binds with higher affinity to sterols and/or Insigs than reductase. When Insig-1 is present (as in sterol-depleted cells), SCAP-SREBP binding occurs rapidly upon sterol addition, and nuclear SREBP declines within 5 h. When Insig-1 is selectively absent (as in SRD-14 cells), the cells require a longer time to accumulate sufficient sterols to trigger binding to Insig-2. Hence, nuclear SREBP-2 does not disappear until 16 h after sterol addition. The conclusion that this delayed inhibition of SREBP cleavage is supported by the finding that the delayed response is abolished in SRD-15 cells, which have a severe reduction in Insig-2 as well as Insig-1. Because they lack both rapid and delayed responses, the SRD-15 cells (unlike the SRD-14 cells) are able to grow continuously in the presence of 25-hydroxycholesterol (see Fig. 1).

Until now, two types of mutant CHO cells have been classified as resistant to 25-hydroxycholesterol in that they fail to suppress cholesterol synthesis under conditions of sterol overload and survive chronic oxysterol treatment (31). Type 1 mutants express truncated forms of SREBP-2 that constitutively migrate to the nucleus and activate gene transcription, regardless of sterol treatment. Study of the type 1 mutants revealed the central role of SREBP-2 in cholesterol homeostasis and provided genetic evidence for the regulated proteolytic pathway by which SREBP activation is controlled (21). Type 2 sterol-resistant CHO cells harbor activating point mutations in the sterol-sensing domain of SCAP. These mutations render mutant SCAP refractory to sterol regulation; therefore, it escorts SREBPs from the ER to the Golgi even when the cells are overloaded with sterols. Studies of the type 2 mutants revealed the existence of SCAP and identified its activity as the target of sterol-mediated regulation of SREBP processing (32). It should be noted that both type 1 and type 2 sterol-resistant mutants exhibit a dominant phenotype.

Data in the current paper demonstrate that the SRD-15 cells represent a new type of mutant CHO cells designated as type 3 sterol-resistant mutants. The underlying defect that renders SRD-15 cells type 3 sterol-resistant mutants has been traced to their deficiencies in Insig-1 and Insig-2, and the current studies establish an essential role for both Insig-1 and Insig-2 in the sterol regulation of SREBP processing and reductase degradation. However, unlike other sterol-resistant mutants, the phenotype of the type 3 mutants is recessive because of their lack of both Insig-1 and Insig-2. Future studies with these cells may provide valuable insights into other aspects of the cellular maintenance of lipid homeostasis. For example, the SRD-15 cells provide an ideal context in which to evaluate the possible participation of Insigs in other aspects of the cellular domain-containing proteins.

Acknowledgments—We thank Drs. Michael S. Brown and Joseph L. Goldstein for their continued encouragement and critical reading of the manuscript. We also thank Tammy Dinh and Kristi Garland for excellent technical support, Dr. Robert Rawson for help with mutagenesis, and Lisa Beatty for invaluable help with tissue culture.

REFERENCES


Downloaded from http://www.jbc.org/ by guest on November 18, 2017
Isolation of Sterol-resistant Chinese Hamster Ovary Cells with Genetic Deficiencies in Both Insig-1 and Insig-2
Peter C. W. Lee, Navdar Sever and Russell A. DeBose-Boyd

doi: 10.1074/jbc.M502989200 originally published online May 2, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502989200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 31 references, 20 of which can be accessed free at http://www.jbc.org/content/280/26/25242.full.html#ref-list-1