Sterol-regulated Degradation of Insig-1 Mediated by the Membrane-bound Ubiquitin Ligase gp78*

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Insig-1 and Insig-2, closely related endoplasmic reticulum membrane proteins, mediate transcriptional and post-transcriptional mechanisms that assure cholesterol homeostasis through their sterol-induced binding to Scap (SREBP cleavage-activating protein) and 3-hydroxy-3-methylglutaryl coenzyme A reductase. Recent studies show that Insig-1 (but not Insig-2) is ubiquitinated and rapidly degraded when cells are depleted of sterols. Conversely, ubiquitination of Insig-1 is blocked, and the protein is stabilized when intracellular sterols accumulate. Here, we report that the ubiquitin ligase gp78, which binds with much higher affinity to Insig-1 than Insig-2, is required for ubiquitination and degradation of Insig-1 in sterol-depleted cells. Sterols prevent Insig-1 ubiquitination and degradation by displacing gp78 from Insig-1, an event that results from sterol-induced binding of Scap to Insig-1. In addition to providing a mechanism for sterol-regulated degradation of Insig-1, these results help to explain why Scap is subject to endoplasmic reticulum retention upon Insig-1 binding, whereas 3-hydroxy-3-methylglutaryl coenzyme A reductase is ubiquitinated and degraded.

The endoplasmic reticulum (ER) membrane proteins Insig-1 and Insig-2 coordinate cholesterol synthesis through their sterol-induced binding to the Scap (SREBP cleavage-activating protein) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (1). Scap controls the activation of SREBPs, membrane-bound transcription factors that enhance transcription of genes required for cholesterol synthesis and uptake (2, 3). Binding of Scap to Insig proteins leads to ER retention of Scap, thereby preventing delivery of Scap-bound SREBPs to the Golgi for proteolytic release from membranes (4, 5). In the absence of this proteolytic activation, transcriptional rates of SREBP target genes decline, leading to a reduction in cholesterol synthesis and uptake. Sterol-induced binding of HMG CoA reductase to Insig proteins leads to the ubiquitination and subsequent degradation of the reductase by 26 S proteasomes (6, 7). This degradation slows the reductase-catalyzed conversion of HMG CoA to mevalonate, a rate-limiting step in cholesterol synthesis (8). In combination, these sterol-mediated actions of Insig proteins (ER retention of Scap and degradation of HMG CoA reductase) prevent overaccumulation of cholesterol or its sterol precursors.

Human Insig-1 and Insig-2 are 59% identical and share a similar topology with both proteins containing six transmembrane helices (5, 9). Despite these similarities and overlapping functions in sterol regulation, Insig-1 and Insig-2 differ in their mode of regulation. The Insig-1 gene is a target of nuclear SREBPs. Thus, Insig-1 mRNA levels are high in sterol-deprived cells and decline under sterol-replete conditions (3, 4). The Insig-2 transcript is expressed constitutively in cultured cells and is not regulated by nuclear SREBPs (5). Paradoxically, the level of Insig-1 protein varies opposite to that of its mRNA. In sterol-depleted cells in which Insig-1 mRNA levels are high, the Insig-1 protein becomes ubiquitinated and rapidly degraded by proteasomes with a half-life <30 min (10). When cells are presented with sterols, Scap binds to Insig-1, and this event blocks Insig-1 ubiquitination, thereby extending its half-life to >2 h (10). Thus, the steady state levels of Insig-1 protein remain the same despite a reduction in its mRNA (10). In contrast to the results with Insig-1, Insig-2 has a much longer half-life that is not subject to sterol regulation (11, 12). These two levels of Insig-1 regulation (gene transcription and protein stability) are central to a process recently termed “convergent feedback inhibition” (10). When cells are depleted of sterols, the Scap-SREBP complex dissociates from Insig-1, which in turn becomes ubiquitinated and degraded. This allows translocation of Scap-SREBP complex to the Golgi, where SREBPs are processed to the nuclear form that accelerates transcription of genes required for cholesterol synthesis and uptake. Transcription of the Insig-1 gene is also enhanced, which leads to an increase in the synthesis of Insig-1 protein. However, this protein will be rapidly degraded until sufficient sterols accumulate to stimulate Scap binding to Insig-1. Thus, inhibition of SREBP processing requires the convergence of newly synthesized Insig-1 and newly acquired cholesterol, thereby preventing premature termination of SREBP activation.

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4 The abbreviations used are: ER, endoplasmic reticulum; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LPDS, lipoprotein-deficient serum; CMV, cytomegalovirus; GFP, green fluorescent protein; HA hemagglutinin; TK, thymidine kinase; RNAi, RNA interference; CFTR, cystic fibrosis transmembrane conductance regulator.
Insight into the mechanism for convergent feedback inhibition may be provided by the recent discovery of gp78 as an Insig-1-associated protein (13). The membrane-bound gp78 is a RING finger ubiquitin ligase (E3) that mediates ubiquitination of reductase in the presence of sterols (13). In the current study, we expand upon this observation by examining a possible role for gp78 in sterol-regulated ubiquitination and degradation of Insig-1. Our results show that gp78 is required for the ubiquitination and degradation of Insig-1 in sterol-depleted cells. Sterol-dependent stabilization of Insig-1 occurs when the protein binds to Scap, which in turn displaces gp78, thereby preventing ubiquitination and subsequent degradation of Insig-1. These results explain the sterol-regulated stability of Insig-1 protein and provide a new model for the underlying mechanism of convergent feedback inhibition.

EXPERIMENTAL PROCEDURES

Materials—MG-132 and Nonidet P-40 alternative (Nonidet P-40) were obtained from Calbiochem, Fos-Choline 13 was obtained from Anacatre,; hydroxypropyl-β-cyclodextrin was obtained from Cyclodextrin Technologies, and polyclonal anti-Myc, anti-hemagglutinin (HA), and anti-T7 IgG were obtained from Bethyl Laboratories. Cycloheximide and monoclonal anti-hemagglutinin (HA) IgG was obtained from Upstate Biotechnology, horse-radish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgGs (affinity-purified) were obtained from Jackson ImmunoResearch, and hybridoma cells producing IgG-9E10, a mouse monoclonal antibody against Myc tag, were obtained from the American Type Culture Collection (Manassas, VA). IgG-R139, a rabbit polyclonal antibody against hamster Scap has been described previously (14). A polyclonal antibody against human gp78 was generated by immunizing rabbits with a recombinant protein consisting of glutathione against human gp78 was generated by immunizing rabbits with a recombinant protein consisting of glutathione transferase in Scap. Monolayers of SRD-13A cells were grown at 37 °C in 8% CO2 in tissue culture for the indicated time.

Plasmid Constructs—The following plasmids were described as the indicated reference or obtained from the indicated sources: pTK-Insig1-Myc encoding wild-type human Insig-1 followed by six tandem copies of a c-Myc epitope tag (EQQKLI-SEEDL) under control of the thymidine kinase (TK) promoter (10); pCMV-Insig1-T7, pCMV-Insig2-T7, and pCMV-Insig2(T214A)-T7 encoding full-length versions of wild-type human Insig-1, wild-type human Insig-2, and mutant human Insig-2, respectively, followed by three tandem copies of a T7 epitope tag under control of the cytomegalovirus (CMV) enhancer/promoter (12); pEF1a-HA-ubiquitin (provided by Dr. Zhijian Chen, University of Texas Southwestern Medical Center) encodes amino acids 1–76 of human ubiquitin preceeded by an epitope tag derived from the influenza HA protein (YPYDVPDY) under the control of the EF1a promoter; pCMV-Scap and pCMV-Scap(Y298C) encoding wild-type and mutant hamster Scap, respectively, under control of the CMV promoter (18); pClneo-gp78 (obtained from Dr. Allan M. Weis-sman, National Cancer Center) encoding human gp78 under the control of the CMV promoter (19); and pCMV-CFTR(ΔF508) (provided by Dr. Philip J. Thomas, University of Texas Southwestern Medical Center) encoding a mutant cystic fibrosis transmembrane conductance regulator that contains a deletion of phenylalanine at position 508 under control of the CMV promoter.

Tissue Culture Medium—Medium A contained 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% (v/v) newborn calf LPDS, 50 μM sodium compactin, and 50 μM sodium mevalonate. Medium C contained 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 D contained medium C supplemented with 5% (v/v) newborn calf LPDS, 50 μM sodium compactin, and 50 μM sodium mevalonate.

Cell Culture—Monolayers of SV-589 cells, an immortalized line of human fibroblasts expressing the SV40 large T antigen (21), were grown at 37 °C in 5% CO2. Stock cultures of SV-589 cells were maintained in medium A supplemented with 10% (v/v) fetal calf serum. SRD-14/pTK-Insig1-Myc cells are mutant CHO cells lacking endogenous Insig-1 that stably express with pTK-Insig1-Myc (10). Monolayers of SRD-14/pTK-Insig1-Myc cells were grown at 37 °C in 8% CO2 in medium C supplemented with 5% (v/v) LPDS and 500 μM g418. SRD-13A cells are a clone of mutant CHO cells deficient in Scap. Monolayers of SRD-13A cells were grown at 37 °C in 8% CO2 and maintained in medium C supplemented with 5% (v/v) fetal calf serum, 5 μg/ml cholesterol, 1 mm sodium mevalonate, and 20 μM sodium olate.

Transient Transfection of Cells and Immunoblot Analysis—SRD-13A and SV589 cells were transiently transfected with FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocol. Conditions of incubation after transfection are described in the figure legends. After incubation, duplicate dishes of cells were pooled, harvested, and lysed in 0.1 ml of buffer A (25 mM Tris-Cl at pH 7.2, 0.15 M NaCl, 1% (v/v) Nonidet P-40, a protease inhibitor mixture including 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 μg/ml aprotinin, and 25 μg/ml N-acetyl-leucinal-leucinal-norleucinal). Aliquots of the lysate were subjected to SDS-PAGE and immunoblot analysis. Antibodies used in the current studies were IgG-R139 (5 μg/ml), IgG-9E10 (1 μg/ml), a polyclonal anti-Myc (0.2 μg/ml), a polyclonal anti-T7 (0.2 μg/ml), a monoclonal anti-HA IgG (1:1,000 dilution), a polyclonal anti-gp78 (1:10,000 dilution), a monoclonal anti-CFTR (1:1,000 dilution), and horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgGs (0.2 μg/ml). Bound antibodies were visualized by chemiluminescence using the SuperSignal substrate system (Pierce) according to the manufacturer’s instructions. Filters were exposed to Kodak X-Omat Blue XB-1 films at room temperature for the indicated time.

RNA Interference (RNAi)—Duplexes of small interfering RNA (siRNA) were synthesized by Dharmacon Research (Lafayette, CO). The three siRNA sequences targeting human gp78 (GenBank™ accession number NM_001144) are at nucleotide positions (relative to the codon for the initiating chain)
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**RESULTS**

Fig. 1 shows an experiment that addresses the requirement of gp78 for degradation of Insig-1 in sterol-depleted SV-589 cells, a line of immortalized human fibroblasts (21). Inasmuch as we do not have an antibody capable of detecting endogenous Insig-1 in human cells, SV-589 cells were transfected with an expression plasmid that encodes human Insig-1 tagged with six copies of the c-Myc epitope. Expression of the transfected Insig-1 is driven by the weak thymidine kinase promoter to prevent saturation of the regulated degradation machinery. The cells were subsequently transfected with duplexes of siRNA targeting either gp78 or green fluorescent protein (GFP), a control mRNA not present in the cells. Following incubation in medium containing sterols, which prevents ubiquitination and degradation of Insig-1 (10), the cells were switched to sterol-depleting medium and treated with cycloheximide to block the generation of newly synthesized Insig-1. At various times after cycloheximide treatment, cells were harvested for lysate preparation and immunoblot analysis with anti-Myc. In previous studies, we found that the time course of Insig-1 disappearance following cycloheximide treatment reflects its rate of degradation (10). In cells receiving the GFP siRNA, Insig-1 declined by >50% after 1 h and became barely detectable after 2 h of cycloheximide treatment (Fig. 1A, lanes 2–4). This disappearance was significantly blunted by transfection of three siRNAs that target different regions of the gp78 mRNA, with >50% of Insig-1 remaining in the cells after 2 h of cycloheximide treatment (Fig. 1A, lanes 5–13). Endogenous Myc protein was also detected in the same immunoblot, because anti-Myc was used to detect transfected Insig-1. The disappearance of Myc protein after cycloheximide treatment was not affected by transfection of siRNAs targeting gp78 (Fig. 1A, lanes 2–13). Quantitative real-time PCR revealed that each gp78 siRNA duplex decreased the level of gp78 mRNA by >80% (Fig. 1B). Notably, the degradation of Insig-1 was not completely abolished in cells transfected with siRNA targeting

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**FIGURE 1.** RNAi-mediated knockdown of gp78 blocks degradation of Insig-1 in sterol-depleted cells. On day 0, SV589 cells were set up at 2.1 × 10⁶/60-mm dish. On day 1, cells were transfected with 0.2 μg of pTK-insig1-Myc. The amount of DNA in each dish was adjusted to 1.0 μg by the addition of empty vector pcDNA3.1. Following incubation for 5 h at 37 °C, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or different regions of gp78 (gp78-1, gp78-2, and gp78-3). On day 2, cells were switched to medium A supplemented with 5% LPDS, 10 μM/ml of cholesterol, and 1 μM/ml of 25-hydroxycholesterol. A, on day 3, cells were treated with 1% (w/v) hydroxypropyl-β-cyclodextrin in sterol-depleting medium B for 1 h, after which they were washed and incubated in sterol-depleting medium B containing 50 μM cycloheximide (CHX). After the indicated period of time, cells were harvested, and detergent lysates were prepared and subjected to SDS-PAGE followed by immunoblot analysis with anti-Myc IgG-9E10 (against Insig-1). The filter was exposed for 10 s. B, on day 3, total RNA was prepared from cells and subjected to first strand cDNA synthesis and quantitative real-time PCR analysis. Each value for cells transfected with the indicated siRNA represents the amount of gp78 mRNA relative to that in control cells transfected with GFP siRNA.

methionine) 300–318, 1130–1318 and 1318–1336 for gp78-1, gp78-2, and gp78-3, respectively. The siRNA sequences targeting human Hrd1 and Trc8 (GenBank™ accession numbers AF317634 and AF064801, respectively) correspond to the following nucleotide positions relative to the codon for the initiating methionine Hrd1 (nucleotides 177–195) and Trc8 (nucleotides 1663–1681). SV589 cells cultured in a 60-mm dish were transfected with 400 pmol of siRNA duplexes using OligofectAMINETM reagent (Invitrogen) as described by the manufacturer, after which the cells were used for experiments as described in the figure legends.

**Real-time PCR**—The protocol was identical to that described by Liang et al. (20). Briefly, triplicate samples of first strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with human 36B4 as an invariant control. Relative amounts of mRNAs were calculated using the comparative C_T method.

**Immunoprecipitation**—The pooled cell pellets from duplicate 60-mm dishes of cells were lysed and immunoprecipitated as described previously (14).

**Ubiquitination of Insig-1**—Cells were directly lysed in the dish by the addition of buffer B (25 mM Tris-HCl at pH 7.2, 0.15 M NaCl, 0.5% (v/v) Fos-choline 13, 10 mM N-ethylmaleimide, a protease inhibitor mixture including 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 μg/ml aprotinin, and 25 μg/ml N-acetyl-leucin-leucin-norleucinal) supplemented with 8 M urea. The cell lysates were sonicated for 5 s at 4 °C with a Branson Digital Sonifier. The resulting cell lysates were diluted with buffer B to decrease the urea concentration to 2 M and subjected sequentially to immunoprecipitation and immunoblot analysis to determine the ubiquitination of Insig-1 as previously described (10).
gp78. This is most likely due to the activity of residual gp78 remaining in the cell after RNAi treatment. As an E3 ubiquitin ligase, gp78 acts catalytically rather than stoichiometrically. Thus, a small amount of gp78 could still catalyze the ubiquitination of fractions of Insig-1 resulting in their degradation.

A similar RNAi experiment was conducted to demonstrate the requirement of gp78 for Insig-1 ubiquitination (Fig. 2). SV589 cells were transfected with a plasmid encoding Insig-1 fused to three copies of the T7 epitope and a plasmid encoding gp78. The cells were depleted of sterols and incubated in sterol-depleting medium B for 1 h, after which they were washed and incubated in sterol-depleting medium B in the absence (−) or presence (+) of 10 μM MG-132. After incubation for 2 h, cells were harvested, and detergent lysates were prepared, and immunoprecipitation was carried out with anti-T7-coupled agarose beads and immunoblot analysis with anti-HA IgG (against ubiquitin) and polyclonal anti-T7 IgG (against Insig-1). Aliquots of the cell lysates were removed prior to anti-T7 precipitation and subjected to SDS-PAGE and immunoblot analysis with anti-gp78. The filters were exposed to film for 5 s.

![Image](image1.png)

**FIGURE 2.** RNAi-mediated knockdown of gp78 prevents ubiquitination of Insig-1 in sterol-depleted cells. SV589 cells were set up and transfected with 0.2 μg of pEF1a-HA-ubiquitin and 0.2 μg of pCMV-Insig1-T7 as described in the legend to Fig. 1. On day 1, 5 h after DNA transfection, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or gp78 (gp78-2). On day 3, cells were treated with 1% (w/v) hydroxypropyl-β-cyclodextrin in sterol-depleting medium B for 1 h, after which they were washed and incubated in sterol-depleting medium B in the absence (−) or presence (+) of 10 μM MG-132. After incubation for 2 h, cells were harvested, and detergent lysates were subjected sequentially to immunoprecipitation with monoclonal anti-T7 IgG-coupled agarose beads and immunoblot analysis with anti-HA IgG (against ubiquitin) and polyclonal anti-T7 IgG (against Insig-1). Aliquots of the cell lysates were removed prior to anti-T7 precipitation and subjected to SDS-PAGE and immunoblot analysis with anti-gp78. The filters were exposed to film for 5 s.

![Image](image2.png)

**FIGURE 3.** Specificity for gp78 in mediating Insig-1 degradation. A, SV589 cells were set up and transfected with 0.6 μg of pTK-Insig1-myc or 1.0 μg of pCMV-CFTR(ΔF508) as described in the legend to Fig. 1. The total amount of DNA in each dish was adjusted to 1.0 μg by the addition of empty vector pcDNA3.1. On day 1, 5 h subsequent to DNA transfection, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or gp78 (gp78-2). On day 2, cells were switched to medium A containing 5% LPDS with 10 μg/ml of cholesterol and 1 μg/ml of 25-hydroxycholesterol. On day 3, cells were treated with 1% (w/v) hydroxypropyl-β-cyclodextrin in sterol-depleting medium B for 1 h and then washed and incubated in sterol-depleting medium B containing 50 μM cycloheximide (CHX) for the indicated time. Cells were then harvested and cell lysate was subjected to SDS-PAGE and immunoblot analysis with anti-Myc IgG-9E10 (against Insig-1) and monoclonal anti-CFTR. Filters were exposed to film for 1 min. B and C, SV589 cells were set up and transfected with 0.6 μg of pTK-Insig1-myc as described in the legend to Fig. 1. Five h after DNA transfection, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP, gp78, Trc8, or Hrd1 as indicated. On day 2, cells were switched to medium A containing 5% LPDS with 10 μg/ml of cholesterol and 1 μg/ml of 25-hydroxycholesterol. On day 3, cells were treated, harvested, and subjected to immunoblot analysis with anti-Myc IgG-9E10 (against Insig-1) and monoclonal anti-CFTR. Filters were exposed to film for 1 min. B and C, SV589 cells were set up and transfected with 0.6 μg of pTK-Insig1-myc as described in the legend to Fig. 1. Five h after DNA transfection, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP, gp78, Trc8, or Hrd1 as indicated. On day 2, cells were switched to medium A containing 5% LPDS with 10 μg/ml of cholesterol and 1 μg/ml of 25-hydroxycholesterol. On day 3, cells were transfected, harvested, and subjected to immunoblot analysis with anti-Myc IgG-9E10 (against Insig-1) and monoclonal anti-CFTR. Filters were exposed to film for 1 min. B and C, SV589 cells were set up and transfected with 0.6 μg of pTK-Insig1-myc as described in the legend to Fig. 1. Five h after DNA transfection, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP, gp78, Trc8, or Hrd1 as indicated.
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**A**

| IP fractions | Pellet | Sup. |
|--------------|--------|------|
| Sterols      | –      | +    |
| Lane         | 1 2 3 4 |

**B**

| IP fractions | Pellet | Sup. |
|--------------|--------|------|
| pCMV-SCAP    | –      | –    |
| pCMV-Insig1-T7 | –  | –  |
| pClneo-gp78  | –      | +    |
| Sterols      | –      | +    |
| Lane         | 1 2 3 4 5 6 |

**C**

| IP fractions | Pellet | Sup. |
|--------------|--------|------|
| pCMV-SCAP    | –      | –    |
| pCMV-Insig1-T7 | –  | –  |
| pClneo-gp78  | –      | +    |
| Lane         | 1 2 3 4 5 6 |

FIGURE 4. Association of gp78 and Insig-1 is blocked by sterols in a reaction that requires Scap. A, on day 0, SRD-14/pTK-Insig1-Myc cells were set up at 3.5 × 10⁵ cells/60-mm dish. On day 2, cells were switched into sterol-depleting medium D. On day 3, cells were incubated with medium D in the absence (−) or presence (+) of 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol. After incubation for 5 h, cells were harvested, lysed, and immunoprecipitated (IP) with polyclonal anti-Myc to precipitate Insig-1. Pellets (representing a 0.25 dish of cells) and supernatants (representing a 0.05 dish of cells) of the immunoprecipitation were subjected to SDS-PAGE and immunoblot (IB) analysis with anti-Myc IgG-9E10 (against Insig-1) and anti-gp78. The filters were exposed to film for 2–60 s. B, on day 0, SRD-13A cells were set up at 3.5 × 10⁵ cells/60-mm dish. On day 2, cells were transfected with 0.5 μg of pCMV-Scap, 0.03 μg of pCMV-Insig1-T7, and 0.3 μg of pClneo-gp78 as indicated. The total amount of DNA in each dish was adjusted to 2.0 μg by the addition of empty vector pcDNA3.1. On day 3, the cells were treated with 1% (w/v) hydroxypropyl-β-cyclodextrin in sterol-depleting medium D for 1 h and then washed and switched to medium D in the absence (−) or presence (+) of 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol. After incubation for 5 h, cells were harvested, lysed, and immunoprecipitated with monoclonal anti-T7 IgG-coupled agarose beads to precipitate Insig-1. Pellets (representing a 0.25 dish of cells) and supernatants (representing a 0.05 dish of cells) of the immunoprecipitation were subjected to SDS-PAGE and immunoblot analysis with polyclonal anti-T7 (against Insig-1), IgG-R139 (against Scap), and anti-gp78. The filters were exposed to film for 2–60 s.

panel, lane 5). Constant amounts of Insig-1 were found in immunoprecipitates from MG-132-treated cells, regardless of the siRNA duplex transfected (Fig. 2, middle panel, lanes 4 and 5). Notably, we did not observe the appearance of a smear of ubiquitinated Insig-1 in the anti-T7 immunoblot. This likely results from rapid debiquitination of Insig-1 because of the strong activity of debiquitinating enzymes in the lysates (7, 22). Taken together, these results indicate that gp78 is required for ubiquitination of Insig-1 in sterol-depleted cells.

Fig. 3 shows experiments that address the specificity of gp78 in mediating degradation of Insig-1. We first examined whether RNAi-induced knockdown of gp78 retards degradation of another polytopic protein from the ER membrane. For this purpose, we transfected SV589 cells with an expression plasmid encoding Insig-1 or a mutant version of the cystic fibrosis transmembrane conductance regulator protein CFTR(ΔF508), a well known substrate for ER-associated degradation (23). The cells were then transfected with the control or gp78 siRNA duplex, incubated in sterol-depleting medium, and subjected to cycloheximide chase analysis. As expected, Insig-1 degradation was slowed in cells transfected with the gp78 but not the control siRNA duplex (Fig. 3A, upper panel). However, CFTR(ΔF508) continued to be rapidly degraded, even in cells that received the gp78 siRNA duplex (Fig. 3A, bottom panel).

We next designed an experiment to determine whether degradation of Insig-1 in sterol-depleted cells requires the presence of other membrane-bound putative sterol-sensing domain (24), and Hrd1, a homolog of gp78. Although Saccharomyces cerevisiae Hrd1p is required for regulated degradation of the yeast reductase isozyme HMG2p (25), its mammalian ortholog neither mediates regulated degradation of mammalian reductase nor binds Insig-1 (13). To determine whether Hrd1 or Trc8 are required for degradation of Insig-1, their expression was reduced in SV-589 cells by RNAi (Fig. 3B). In contrast to the result of 22% reduction of Insig-1, RNAi-mediated knockdown of Hrd1 and Trc8 did not block degradation of Insig-1 in sterol-depleted cells (Fig. 3B, lanes 6–9). Quantitative real-time PCR revealed that mRNAs for gp78, Hrd1, and Trc8 were reduced by >70% in the experiment (Fig. 3C).

Inasmuch as ubiquitination and degradation of Insig-1 is blocked by sterols (10), we next examined whether binding...
between gp78 and Insig-1 is similarly blocked by sterols. To avoid potential artifacts generated by overexpression, we analyzed SRD-14/pTK-Insig-1-Myc cells, mutant CHO cells that lack endogenous Insig-1 but stably express Myc-tagged Insig-1 at levels that are similar to those found in wild-type cells (10). The cells were cultured in the absence or presence of sterols, after which they were harvested, and Insig-1 was immunoprecipitated from lysates by anti-Myc. Immunoblot analysis of the resulting immunoprecipitates revealed that a small fraction of endogenous gp78 co-immunoprecipitated with Insig-1 when the cells were cultured in the absence of sterols (Fig. 4A, lane 1). Incubation of cells with sterols increased the amount of Insig-1, but decreased the amount of gp78 co-immunoprecipitated with Insig-1 (Fig. 4A, lane 2). It should be noted that the presence of a large fraction of gp78 not bound to Insig-1 likely reflects a role for the E3 in processes unrelated to Insig-1 degradation (19, 26, 27).

In previous studies, we found that sterol-induced binding of Scap to Insig-1 is required for sterols to block Insig-1 ubiquitination (10). Thus, an experiment was designed to determine whether Scap is required for sterol-mediated inhibition of gp78-Insig-1 complex formation. SRD-13A cells, a line of mutant CHO cells lacking Scap (28), were transfected with expression plasmids encoding Scap, gp78, and T7-tagged Insig-1. The cells were then incubated in the absence or presence of sterols, after which they were harvested, lysed, and Insig-1 was immunoprecipitated with anti-T7 beads. Immunoblotting the precipitates revealed that, in the absence of Scap, gp78 was co-immunoprecipitated with Insig-1 in the absence or presence of sterols (Fig. 4B, lanes 3 and 4). Co-expression of Scap stabilized Insig-1 even in the absence of sterols (Fig. 4B, lane 5, bottom panel), and this correlated with a trace amount of Scap in the precipitate (Fig. 4B, lane 5, middle panel). In precipitates from the same cell lysates, binding between gp78 and Insig-1 was slightly inhibited (Fig. 4B, lane 5, top panel). Treatment with sterols further stabilized Insig-1 (Fig. 4B, lane 6, bottom panel); more Scap and less gp78 were co-precipitated with Insig-1 (Fig. 4B, lane 6, middle and top panels). These results indicate that sterol-mediated dissociation between gp78 and Insig-1 requires Scap, and this event correlates with stabilization of Insig-1.

To further confirm that Scap binding to Insig-1 is required for the sterol-mediated inhibition of gp78-Insig-1 complex formation, we co-transfected SRD-13A cells with an expression plasmid encoding Scap(Y298C), which is defective in binding to Insig-1 (29). Transfected cells were cultured in the presence of sterols, and the amount of gp78 and Scap that co-immunoprecipitated with Insig-1 was determined as described in the experiments of Fig. 4B. As shown in Fig. 4C, wild-type Scap co-immunoprecipitated with Insig-1, and this interaction led to reduced binding between gp78 and Insig-1 (Fig. 4C, lanes 3–5). In contrast, Scap(Y298C) did not co-immunoprecipitate with Insig-1 (Fig. 4C, lanes 6–7) and had no effect on the amount of gp78 co-immunoprecipitated with Insig-1 (Fig. 4C, lanes 3, 6, and 7).

Fig. 5 shows an experiment in which we examined the binding between gp78 and Insig-2, which is not subject to rapid degradation in the absence of sterols (12). SRD-13A cells were transfected with expression plasmids encoding gp78 and T7-tagged Insig-1 or Insig-2. The cells were cultured in medium containing 5% fetal calf serum prior to preparation of detergent lysates, which were immunoprecipitated with anti-T7 beads to precipitate Insig-1 and -2. Pellets (representing a 0.25 dish of cells) and supernatants (representing a 0.05 dish of cells) of the immunoprecipitation were subjected to SDS-PAGE and immunoblot (IB) analysis with polyclonal anti-T7 (against Insig-1 and -2) and anti-gp78. The filters were exposed to film for 10 s.

**FIGURE 5.** gp78 binds with high affinity to Insig-1 but not Insig-2. SRD-13A cells were set up and transfected with 0.1 μg of pcNeo-gp78, 0.15 μg of pCMV-Insig1-T7, 0.7 μg of wild-type pCMV-Insig2-T7, and 2.5 μg of a mutant version (E214A) as described in Fig. 4B. The total amount of DNA in each dish was adjusted to 3.0 μg by the addition of empty vector pcDNA3.1. On day 3, cells were harvested, lysed, and immunoprecipitated (IP) with monoclonal anti-T7 IgG-coupled agarose beads to precipitate Insig-1 and -2. Pellets (representing a 0.25 dish of cells) and supernatants (representing a 0.05 dish of cells) of the immunoprecipitation were subjected to SDS-PAGE and immunoblot (IB) analysis with polyclonal anti-T7 (against Insig-1 and -2) and anti-gp78. The filters were exposed to film for 10 s.

**DISCUSSION**

Considered together with results from a previous study (10), the current data establish a model for sterol-regulated degradation of Insig-1 protein. The essential features of this model are diagrammed in Fig. 6. In sterol-depleted cells, Insig-1 binds to a fraction of gp78, which transfers ubiquitin to Insig-1, targeting it for proteasomal degradation. This conclusion is supported by the RNAi experiments of Figs. 1–3, which show a specific requirement for gp78 in the ubiquitination and rapid degradation of Insig-1 that occurs in sterol-depleted cells. In the presence of sterols, Scap binds to Insig-1 in a reaction that displaces gp78 (Fig. 4). We propose that this displacement prevents ubiquitination of Insig-1, which results in the stabilization of the protein. Inasmuch as gp78 is not found associated with the Scap-Insig-1 complex, Scap is not ubiquitinated or degraded but rather is retained in the ER. Other fractions of Insig-1 bind to reductase in sterol-loaded cells. Insig-1 is unlikely to bind
Insig-1 Ubiquitination Mediated by gp78

FIGURE 6. Model for sterol-mediated control of Insig-1 degradation. In sterol-depleted cells, a fraction of gp78 is associated with Insig-1, which leads to the ubiquitination and subsequent proteasomal degradation of Insig-1. In sterol-loaded cells, a portion of Insig-1 binds to Scap, liberating gp78 from Insig-1. In the absence of gp78, Insig-1 is no longer ubiquitinated and degraded, and the Scap-Insig-1 complex remains sequestered in ER membranes. Another portion of Insig-1 binds to HMG CoA reductase in sterol-loaded cells; however, gp78 remains associated with the reductase-Insig-1 complex. This leads to the ubiquitination and proteasomal degradation of reductase. Whether Insig-1 is also ubiquitinated and degraded along with reductase is presently unknown.

The model shown in Fig. 6 also suggests that gp78, Insig-1 are overexpressed to levels similar to those of the endogenous proteins. Studies in the current studies, Scap, Insig-1, and gp78 were optimised (Fig. 4B). In the original study, Insig-1 and gp78 were overexpressed in cells, and their interaction was not inhibited by sterols (13). We reason that endogenous Scap was not present in sufficient quantities to inhibit the binding of gp78 to Insig-1 when the cells were treated with sterols.

Besides sterols, polyunsaturated fatty acids have been shown to inhibit proteolytic activation of SREBP-1, with unknown mechanism (17). It will be interesting to know whether Insig-1 can be stabilized by polyunsaturated fatty acids, and if so, whether the stabilization is mediated by dissociation of gp78 from Insig-1.

In contrast to Insig-1, Insig-2 does not bind to gp78 with high affinity (Fig. 5). The lack of interaction with gp78 may account for the slower degradation of Insig-2. This is supported by results with the E214A mutant of Insig-2, which degrades at a much faster rate than wild-type Insig-2 (12) and binds to gp78 (Fig. 5). If gp78 does not bind directly to Insig-2, then how does Insig-2 mediate the degradation of reductase? Insig-2 is capable of mediating complex formation between gp78 and reductase (13). Moreover, dominant negative versions of gp78 block Insig-2-mediated degradation of reductase in transient transfection assays. We favor a scenario in which gp78 does not bind to Insig-2 alone but binds with high affinity to the reductase-Insig-2 complex. However, we cannot completely rule out the possibility that Insig-2 mediates degradation of reductase through the action of an unidentified E3. Future studies will be aimed toward completely resolving these issues.

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