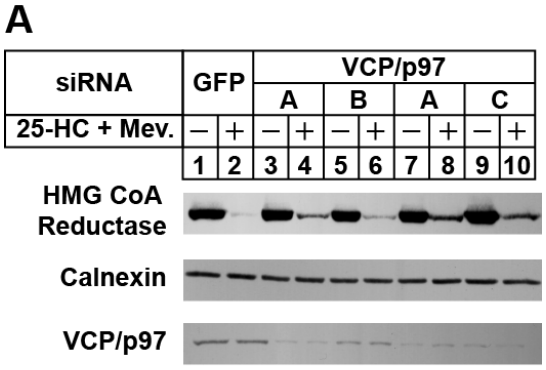
Supplemental Materials

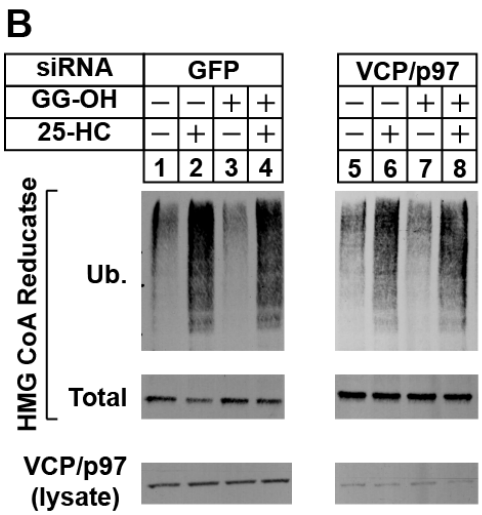
Molecular Biology of the Cell

Elsabrouty et al.

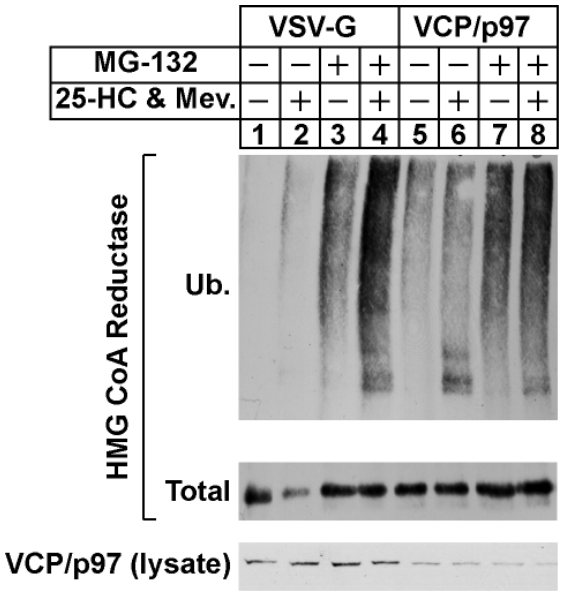
Sterol-Induced Dislocation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase from Membranes of Permeabilized Cells

Rania Elsabrouty, Youngah Jo, Tammy T. Dinh, and Russell A. DeBose-Boyd
From the Howard Hughes Medical Institute and the Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390-9046

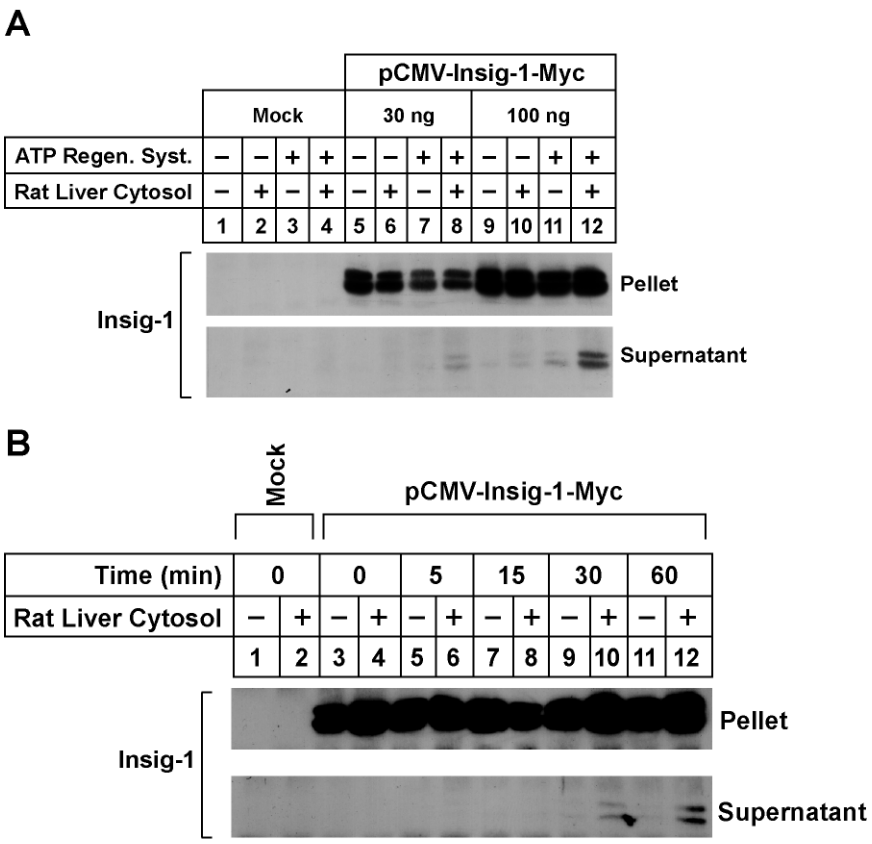




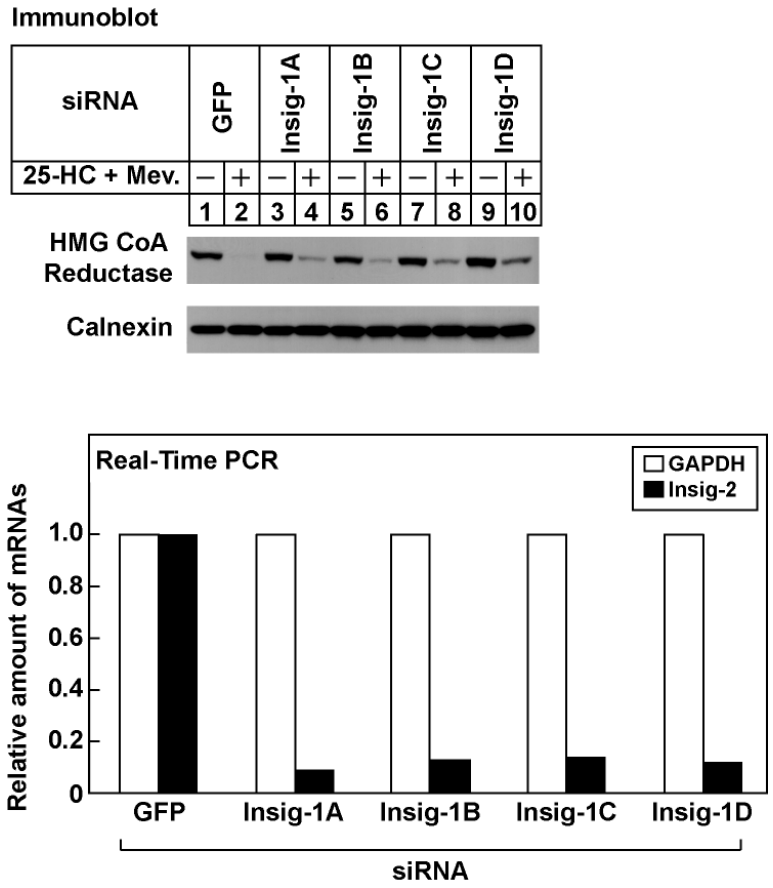
Supplemental Figure 1. Effect of RNAi-mediated knockdown of VCP/p97 on sterol-induced ubiquitination and degradation of HMG CoA reductase in SV-589 cells. SV-589 cells were set up on day 0 at 4 X 10^5 cells per 100-mm dish in medium A supplemented with 10% FCS. On days 1 and 2, the cells were transfected with the indicated siRNAs targeting the control mRNA, green fluorescent protein (GFP), or the VCP/p97 mRNA as indicated. Sequences for the VCP/p97 siRNAs are as follows: A, GAA UAG AGU UGU UCG GAA UUU; B, CCA UCA GCA CUU CGG GAA AUU; and C, GGA GGU AGA UAU UGG AAU UUU. After the second transfection on day 2, cells were depleted of sterols for 16 h at 37 °C and subsequently treated in the absence or presence of 1μg/ml 25-HC plus 10 mM mevalonate (A) or 10 μM geranylgeraniol (GG-OH, B). All of the cells in B received 10 μM MG-132. (A) After 4 h at 37 °C, cells were harvested for preparation of detergent lysates that were subjected to SDS-PAGE and immunoblot analysis with IgG-A9 (against reductase), anti-calnexin IgG, or anti-VCP/p97 IgG. (B) After 2 h 37 °C, detergent lysates were prepared and immunoprecipitation was carried out with polyclonal antibodies against reductase. Aliquots of the immunoprecipitates and total lysates were subjected to SDS-PAGE and immunoblot analysis with IgG-P4D1 (against ubiquitin), IgG-A9 (against reductase), and anti-VCP/p97.



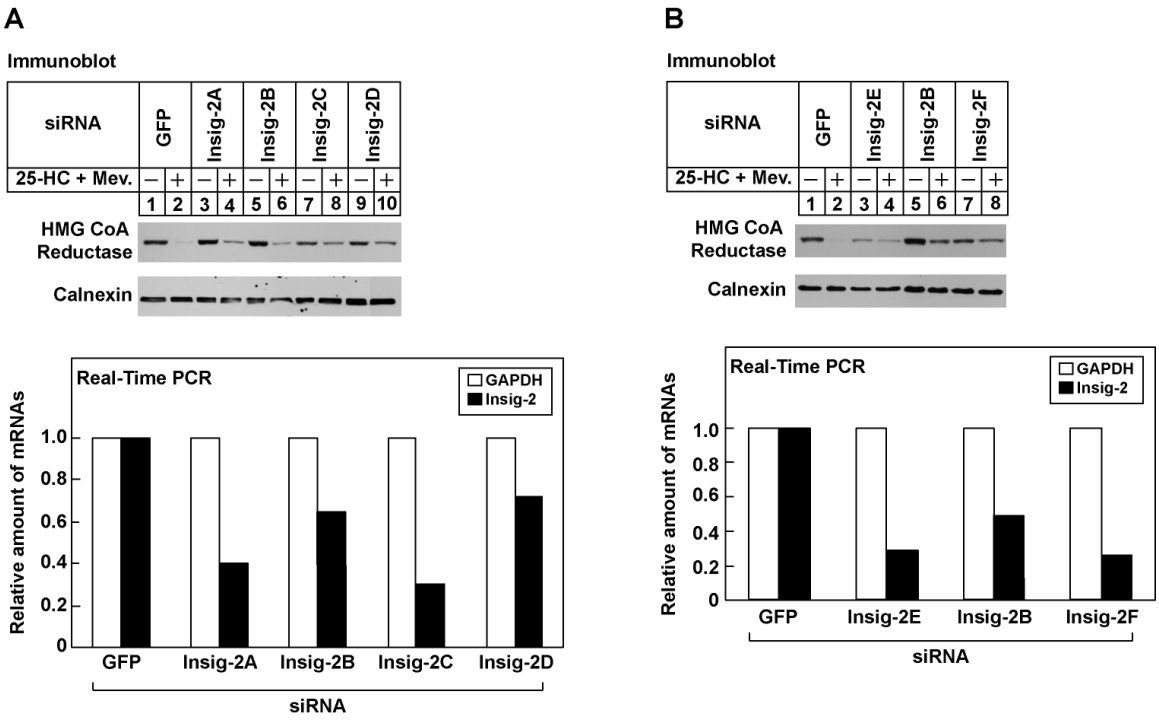
Supplemental Figure 2. RNAi-mediated knockdown of VCP/p97 blunts sterol-accelerated degradation, but not ubiquitination of HMG CoA reductase in Chinese hamster ovary (CHO)-K1 cells. CHO-K1 cells were set up on day 0 at 1 X 10^5 cells per 100-mm dish in a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (medium B) supplemented with 5% FCS. On days 1 and 2, the cells were transfected with the indicated siRNAs targeting the control vesicular stomatitis virus glycoprotein (VSV-G) mRNA or the VCP/p97 mRNA as indicated. The sequence of the VCP/p97 siRNA is as follows: GAA UAG AGU UGU UCG. After the second transfection on day 2, cells were depleted of sterols through incubation in medium B supplemented with 5% LPDS, 10 μM compactin, and 50 μM mevalonate for 16 h at 37 °C and subsequently treated with identical medium containing 10 μM MG-132 in the absence or presence of 1μg/ml 25-HC plus 10 mM mevalonate as indicated. After 4 h 37 °C, detergent lysates were prepared and immunoprecipitation was carried out with polyclonal antibodies against reductase. Aliquots of the immunoprecipitates and total lysates were subjected to SDS-PAGE and immunoblot analysis with IgG-P4D1 (against ubiquitin), IgG-A9 (against reductase), and anti-VCP/p97.



Supplemental Figure 3. Dislocation of Insig-1 from membranes of permeabilized SV-589 cells. SV-589 cells were set up at 2.5 X 10^5 cells per 100-mm dish in medium A containing 10% FCS. On day 3, the cells were transfected with 30-100 ng/dish of pCMV-Insig-1-Myc, which encodes amino acids 1-277 of human Insig-1 followed by six copies of the c-Myc epitope, using the FuGENE6 transfection reagent (Promega, Madison, WI) as previously described (Hartman *et al.*, 2010). The total amount of DNA/dish was adjusted to 4 μg by the addition of pcDNA3.1 mock vector. Six hours following transfection, cells were depleted of sterols for 16 h at 37 °C. The cells were then harvested and permeabilized with 0.025% digitonin as described in the legend to Figure 2. The pellets of permeabilized cells were resuspended in permeabilization buffer containing protease inhibitors and 0.1 mg/ml ubiquitin in the absence or presence of 2 mg/ml rat liver cytosol and the ATP-regenerating system as indicated. Following incubation for 60 min (A) or the indicated period of time (B), the reactions were terminated and samples were subjected to subcellular fractionation. Aliquots of the resulting pellet and supernatant fractions were subjected to immunoblot analysis with IgG-9E10 (against Insig-1).



Supplemental Figure 4. Effect of RNAi-mediated knockdown of Insig-1 on sterol-induced degradation of HMG CoA reductase in SV-589 cells. SV-589 cells were set up on day 0 at 4 X 10^5 cells per 100-mm dish in medium A supplemented with 10% FCS. On days 1 and 2, the cells were transfected with the indicated siRNAs targeting the control mRNA, green fluorescent protein (GFP), or the Insig-1 mRNA as indicated. Sequences for the Insig-1 siRNAs are as follows: A, AATGGTGTCTATCAGTATACA; B, GAACATAGGACGACAGTTA; C, CCCACAAATTTAAGAGAGA; and D, CTGCAGATCCAGAGGAATG. After the second transfection on day 2, cells were depleted of sterols for 16 h at 37 °C and subsequently treated in the absence or presence of 1μg/ml 25-HC plus 10 mM mevalonate. Following incubation for 4 h at 37 °C, cells were harvested for preparation of detergent lysates that were subjected to SDS-PAGE and immunoblot analysis with IgG-A9 (against reductase) and anti-calnexin IgG. Total RNA was prepared from parallel-treated dishes and subjected to reverse-transcription reactions. Triplicate samples of the resulting first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for human Insig-1 and GAPDH. Relative amounts of mRNAs were calculated using the comparative C_T method. Each value for cells transfected with the indicated siRNA represents the amount of Insig-1 mRNA relative to that in control cells transfected with the GFP siRNA.



Supplemental Figure 5. Effect of RNAi-mediated knockdown of Insig-2 on sterol-induced degradation of HMG CoA reductase in SV-589 cells. SV-589 cells were set up on day 0 at 4 X 10^5 cells per 100-mm dish in medium A supplemented with 10% FCS. On days 1 and 2, the cells were transfected with the indicated siRNAs targeting the control mRNA, green fluorescent protein (GFP), or the Insig-2 mRNA as indicated. Sequences for the Insig-1 siRNAs are as follows: A, AATGCGGTGTGTAGCAGTCTT; B, TGACAGACATCTAGGAGAA; C, CTAAAGTGGATTTCGATAA; D, TGGCAATGTACGAATGTAA; E, AGAGAGTGGTCCAGTGTAA; and F, CTTGATGATTCGAGGAGTA (81-99). After the second transfection on day 2, cells were depleted of sterols for 16 h at 37 °C and subsequently treated in the absence or presence of 1μg/ml 25-HC plus 10 mM mevalonate. Following incubation for 4 h at 37 °C, cells were harvested for preparation of detergent lysates that were subjected to SDS-PAGE and immunoblot analysis with IgG-A9 (against reductase) and anti-calnexin IgG. Total RNA was prepared from parallel-treated dishes and subjected to reverse-transcription reactions. Triplicate samples of the resulting first-strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for human Insig-2 and GAPDH. Relative amounts of mRNAs were calculated using the comparative C_T method. Each value for cells transfected with the indicated siRNA represents the amount of Insig-2 mRNA relative to that in control cells transfected with the GFP siRNA.