Characterization of Peroxisomal 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase in UT2* Cells: Sterol Biosynthesis, Phosphorylation, Degradation, and Statin Inhibition†

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ABSTRACT: We have previously identified a CHO cell line (UT2* cells) that expresses only one 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase protein which is localized exclusively in peroxisomes [Engfelt, H. W., Shackelford, J. E., Aboushadi, N., Jessani, N., Masuda, K., Paton, V. G., Keller, G. A., and Krisans, S. K. (1997) *J. Biol. Chem. 272*, 24579−24587]. In this study, we utilized the UT2* cells to determine the properties of the peroxisomal reductase independent of the endoplasmic reticulum (ER) HMG-CoA reductase. We demonstrated major differences between the two proteins. The peroxisomal reductase is not the rate-limiting enzyme for cholesterol biosynthesis in UT2* cells. The peroxisomal reductase protein is not phosphorylated, and its activity is not altered in the presence of inhibitors of cellular phosphatases. Its rate of degradation is not accelerated in response to mevalonate. Finally, the degradation process is not blocked by *N*-acetyl-Leu-Leu-norleucinal (ALLN). Furthermore, the peroxisomal HMG-CoA reductase is significantly more resistant to inhibition by statins. Taken together, the data support the conclusion that the peroxisomal reductase is functionally and structurally different from the ER HMG-CoA reductase.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase is the rate-limiting enzyme in isoprenoid biosynthesis (*1*). It catalyzes the reduction of HMG-CoA to the six-carbon mevalonate, a crucial precursor in the formation of both sterol and non-sterol isoprenoids (*1*). In mammalian cells, HMG-CoA reductase resides in two compartments: the endoplasmic reticulum (ER) and the peroxisomes (*2*−*5*). The ER HMG-CoA reductase is an extensively studied protein due to the critical role it plays in isoprenoid metabolism. Structurally, it is a transmembrane glycoprotein that is composed of two identical 97 kDa subunits (*6*, *7*). The cellular expression and enzymatic activity of the ER HMG-CoA reductase have been shown to be tightly regulated at multiple levels, including transcription, translation, protein stability, and phosphorylation status of the protein (*8*−*13*). The transcriptional regulation of the ER HMG-CoA reductase gene is dependent on a specific interaction between sterol regulatory element binding proteins (SREBPs), which bind to the promoter DNA, and the amino-terminal domain of the transcriptional activator CREB binding protein (CBP) (*14*). When the cellular demand for sterol increases, the transcription of ER HMG-CoA reductase, and other enzymes of the cholesterol and fatty acid biosynthesis pathways, are activated by SREBPs (*15*). In contrast to the well-characterized ER HMG-CoA reductase protein, very little information is available regarding the function and regulation of the peroxisomal HMG-CoA reductase.

To study the role of the peroxisomal HMG-CoA reductase, our group has identified a CHO cell line which expresses only the peroxisomal HMG-CoA reductase (*16*). These cells were obtained by growing UT2 cells (a mutant CHO cell line deficient in the ER HMG-CoA reductase) in the absence of mevalonate (*16*, *17*). The UT2 cells grown in the absence of mevalonate (designated UT2*) were shown to express a 90 kDa HMG-CoA reductase protein which was localized exclusively in peroxisomes (*16*). By contrast, the wild-type CHO cells were shown to contain two HMG-CoA reductase proteins, the well-characterized 97 kDa protein, localized in the ER, and a 90 kDa protein localized in peroxisomes (*16*). In addition, we have demonstrated that the loss of the ER HMG-CoA reductase protein in the UT2 and UT2* cells is due to two point mutations localized in introns of the ER HMG-CoA reductase gene (*18*). One mutation was localized at the 5′ splice junction (+1) position in the intronic sequence between exons 11 and 12, and a second mutation was found at the 5′ splice junction (+5) position in the intronic sequence between exons 13 and 14 (*18*). These mutations result in exon splicing and the insertion of premature stop codons into the mature mRNA transcripts (*18*). The resulting transcripts,

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[1] Abbreviations: CHO, Chinese hamster ovary; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LPDS, lipoprotein deficient serum; FCS, fetal calf serum; DTT, dithiothreitol; ER, endoplasmic reticulum; PMSF, phenylmethanesulfonyl flouride; ALLN, *N*-acetyl-Leu-Leu-norleucinal; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein; CBP, CREB binding protein; FPP, farnesyl diphosphate; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; FACS, fluorescence-activated cell sorter.

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if translated, would produce truncated proteins with molecular masses no larger than 66 kDa. These proteins would be nonfunctional due to the deletion of the catalytic domain of the reductase protein (*18*).

In this study, we utilized the UT2* cells as a model system to investigate the regulation of the 90 kDa peroxisomal HMG-CoA reductase independent of the ER reductase. Specifically, we determined (i) the rate of cholesterol biosynthesis utilizing acetate, pyruvate, mevalonate, and ^3H_2O as substrates, (ii) the activity of the 90 kDa reductase in the presence and absence of inhibitors of cellular phosphatases, (iii) the phosphorylation status of the 90 kDa protein, (iv) the in vitro IC_{50} values of lovastatin, atorvostatin, and simvastatin on peroxisomal HMG-CoA reductase activity, and (v) the effect of mevalonate on reductase activity and the rate of protein synthesis and degradation.

EXPERIMENTAL PROCEDURES

Materials. Biochemicals were purchased from Sigma. Electrophoresis supplies and AG1-X8 200−400 mesh formate resin were purchased from Bio-Rad. All cell culture media and fetal calf serum were purchased from Life Technologies, Inc. Lipoprotein deficient medium was obtained from PerImmune. DL-3-[*glutaryl*-3-^{14}C]-3-Hydroxy-3-methylglutaryl coenzyme A, [1,2-^{3}H(N)]cholesterol, [26-^{14}C]cholesterol, [^{3}H]acetic acid sodium salt, (*RS*)-[5-^{3}H(N)]-mevalonolactone, ^3H_2O, and [2-^{14}C]pyruvic acid sodium salt were purchased from Dupont NEN. [1-^{14}C]Acetyl CoA was purchased from American Radiolabeled Chemicals Inc. Atorvastatin was a generous gift from Park-Davis. Lovastatin and simvastatin sodium salts and HMG-CoA specific inhibitor (L-659,699) were generous gifts from Merck Research Laboratories. UT2 cells were obtained from J. Goldstein.

Cell Culture. CHO and UT2* cells were maintained in 1:1 Dulbecco's modified Eagle's medium/F12, supplemented with 5% fetal calf serum (FCS), fungizone, and pen/strep, in a 37 °C incubator with 5% CO_2. Twenty-four hours prior to all experiments, cells were switched into the same medium supplemented with 5% lipoprotein deficient serum (LPDS). UT2 cells were maintained in the same medium as CHO and UT2* cells but supplemented with 200 μM mevalonate.

Cell Synchronization and Assessment of Cell Survival. UT2 cells were synchronized in the G_0/G_1 interphase of the cell cycle by incubating exponentially growing cultures (1×10^6 cells/plate) for 48 h in complete medium containing 0.5% FCS and 0.2 mM mevalonate. Cell monolayers were washed in salt-balanced buffer [120 mM NaCl and 2.7 mM KCl (pH 7.4)] and supplemented with fresh medium containing 10% FCS. As determined by dye exclusion, after 3 days in medium lacking mevalonate, almost all UT2 cells survived. These UT2 cells were single cell cloned, designated UT2*, and maintained in medium lacking mevalonate.

FACS Analysis. Flow cytometry was used to determine the degree of cell synchronization. Cells were stained with propidium iodine and prepared for fluorescence-activated cell sorter (FACS) analysis by D. Young at the University of California, San Diego, using FACS (Becton Dickinson, Palo Alto, CA). Data were analyzed using multicycle advanced version 3.11 software.

HMG-CoA Reductase Assay. The reaction was carried out as described in ref *16* with the following minor modifications. In HMG-CoA reductase assays where the sodium salts of the statins were tested, the statin salts were dissolved in KPO_4 buffer containing 200 mM NaCl, 30 mM EDTA, and 10 mM DTT (pH 6.8) (KEND buffer) and added to the preincubation mixtures. For the phosphorylation experiments, cells were washed in phosphate-buffered saline (PBS) containing either 50 mM KCl or 50 mM KF, and the cell monolayers were harvested in KEND buffer containing 50 mM KCl or 50 mM KF before measurement of HMG-CoA reductase activity. In addition, to rule out the possibility that we were underestimating the reductase measurements in UT2* cells, Tyloxapol or Nonidet P-40 was substituted for Triton X-100 in the homogenization buffer, freezing−thawing and sonication for cell homogenization, and a number of different thin-layer chromatography solvent systems were employed to separate the product, mevalonate, from its substrate, HMG-CoA. All procedures yielded equivalent results.

HMG-CoA Synthase Assay. The radiochemical assay employed by Brown and Goldstein (*19*) was used to assess the synthesis of [^{14}C]HMG-CoA from [^{14}C]acetyl CoA. For the β-lactone experiments, cells were incubated with the L-659,699 for 3 h prior to HMG-CoA synthase activity measurements being taken.

Determination of the Rate of HMG-CoA Biosynthesis in Vivo. Cells were cultured in 5% LPDS medium containing 5 μM lovastatin and 200 μM mevalonate overnight. Cells were then radiolabeled with 5 μCi of [^{3}H]acetate (1.1×10^5 dpm/nmol) for various periods of time. The rate of HMG-CoA biosynthesis was determined in the presence of 20 000 dpm of [^{14}C]HMG-CoA, used as an internal standard.

Determination of the Rate of HMG-CoA Biosynthesis in Vitro. Cells were cultured in 5% LPDS, scraped in ice-cold 50 mM KPO_4 buffer (pH 7.5), and homogenized in 50 mM KPO_4 buffer containing 0.2% Triton X-100. An aliquot was removed for protein determination, and lovastatin was added to the remaining sample at a final concentration of 5 μM. The cell homogenate was then incubated in the presence of [^{14}C]acetyl CoA (1 μCi/mL) for 1 h at 37 °C.

Acetoacetyl CoA Thiolase Assay. The extent of CoA-dependent cleavage of acetoacetyl CoA was determined spectrophotometrically by measuring the rate of decrease in absorbance at 300 nm as described previously (*20*).

Measurement of the Rate of Cholesterol Biosynthesis. The rate of cholesterol biosynthesis was determined as described previously (*21*) with the following modifications. Cells were grown to 70% confluence on 100 mm plates in standard medium, and transferred to medium containing 5% LPDS 24 h prior to being incubated with the radiolabeled substrates for various periods of time. The radiolabeled substrates included 5 μCi of [^{3}H]acetate (1.1×10^5 dpm/nmol), 50 μCi of [^{3}H]acetate (1.1×10^5 dpm/nmol), 50 μCi of [^{3}H]acetate (1.1×10^4 dpm/nmol), 50 μCi of [^{3}H]mevalonate (1.1×10^4 dpm/nmol), 10 μCi of ^3H_2O, 3.8 μCi of [^{14}C]pyruvate (2.8×10^3 dpm/nmol), or 1 μCi of [^{14}C]acetate (4.4×10^4 dpm/nmol). Where radiolabeled mevalonate was used as the substrate, the medium was supplemented with 2 μM lovastatin for 16 h. For experiments where the substrate was [^{14}C]-pyruvate, cells were washed with PBS and then incubated with buffer A (50 mM Hepes, 100 mM NaCl, 1 mM $CaCl_2$, 10 mM KCl, and 5% LPDS) containing 1 mM glucose and various concentrations of 2-deoxyglucose for 1 h prior to adding the radiolabeled substrate. In the experiments where

the effect of simvastatin on sterol biosynthesis was investigated, cells were grown in 5% LPDS-containing medium overnight. Cells were then incubated in the same medium supplemented with the indicated concentrations of simvastatin for 30 min. After 30 min, 5 μCi of [^{3}H]acetate (1.1×10^5 dpm/nmol) was added and the mixture incubated for 3.5 h before the protocol described in ref *21* was followed.

Immunoprecipitations of HMG-CoA Reductase. The pulse−chase experiments were performed as described previously (*22*). The cells were pulsed for 3 h and chased for various periods of time in complete minimal essential medium (containing 2 mM methionine) with or without 20 mM mevalonate. Aliquots of cell extracts were incubated with anti-HMG-CoA reductase antisera overnight, and immunoprecipitants were isolated on protein A−Sepharose beads. After electrophoresis, proteins were transferred to nitrocellulose and ^{35}S-labeled proteins were visualized with a Molecular Dynamics PhosphorImager system. The immunoprecipitation experiments with [^{35}S]methionine and ^{33}P-radiolabeled HMG-CoA reductase were performed as described previously (*22*). Cells were preincubated with methionine/cysteine-free medium or phosphate-free medium for 1 h, after which either 100−150 μCi of trans ^{35}S label or 300 μCi of ^{33}P label/mL of medium was added and the cells were incubated for 1 or 2 h, respectively. In cells radiolabeled with [^{33}P]phosphate, all buffers used during the washing, harvesting, solubilization, and immunoprecipitation contained phosphatase inhibitors (50 mM KF, 10 mM β-glycerophosphate, and 0.1 mM sodium vandate).

Immunoblotting for HMG-CoA Reductase in the Presence of Mevalonate and ALLN. Cells were incubated overnight in 5% LPDS-supplemented medium, containing 5 μM lovastatin and 200 μM mevalonate. Cells were rinsed three times with PBS and new medium containing 5% LPDS supplemented with 50 μg/mL *N*-acetyl-Leu-Leu-norleucinal (ALLN), or 20 mM mevalonate or 50 μg/mL ALLN with 20 mM mevalonate was added. After incubation for 20 h, cells were rinsed in buffer containing (50 mM KPO_4, 200 mM NaCl, 30 mM EDTA, 10 mM DTT, and 5 mM EGTA) (buffer B) and homogenized in buffer B supplemented with 0.2% Triton X-100, 2 mM PMSF, 100 μM leupeptin, and 2 μg/mL ALLN.

RESULTS

Effect of Cell Synchronization on the Survival Rate of UT2 Cells. In our initial isolation of UT2* cells, we observed a cell survival rate of 25% when a nonsynchronized population of UT2 cells was grown in the absence of mevalonate (*16*). However, the UT2 and UT2* cells were shown to have the same point mutations in the ER HMG-CoA reductase gene (*18*). Since previous studies have reported that the rate of isoprenoid biosynthesis changes as the cells progress through the cell cycle (*23*, *24*), we considered the likelihood that the surviving UT2 cells may be a subset of cells at a unique stage of the cell cycle where the peroxisomal HMG-CoA reductase activity is upregulated and therefore sufficient to meet the cellular demand for mevalonate. Thus, we first questioned the effect of cell synchronization on the survival rate of UT2 cells maintained in 10% FCS medium in the absence of mevalonate.

UT2 cells were synchronized at the G_0/G_1 phase by serum starvation in 0.5% FCS medium supplemented with 200 μM mevalonate for 48 h. Cell synchronization was verified by FACS analysis. At day 0, an equal number of asynchronized and synchronized UT2 cells were switched to 10% FCS medium in the absence of mevalonate, and the cell survival rate was determined over a period of 3 days (Figure 1). As previously observed, only 25% of the asynchronized UT2 cell population survived after 3 days. In contrast, the synchronized UT2 cell population not only survived but also continued to divide. These results suggest that the peroxisomal reductase activity is induced in UT2 cells during the G_0/G_1 phase of the cell cycle. A comparison of HMG-CoA reductase activities of asynchronized and synchronized UT2 cells showed that HMG-CoA reductase activity was 3−4-fold higher in the synchronized cell population (Figure 1). Thus, the data suggest that once the peroxisomal HMG-CoA reductase activity is induced and provides sufficient isoprenoid biosynthesis to support cell replication, the enzyme activity remains upregulated in the UT2* cells maintained in medium lacking mevalonate.

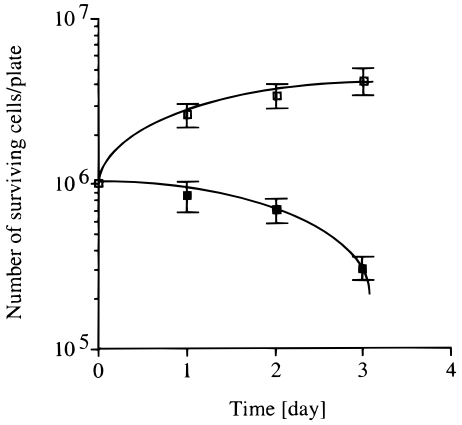

FIGURE 1: Survival rate of asynchronized and synchronized populations of UT2 cells. UT2 cells were synchronized by serum starvation in 0.5% FCS/0.2 mM mevalonate-containing medium for 48 h. Asynchronized UT2 cells were maintained in 5% FCS/0.2 mM mevalonate-supplemented medium. On day 0, asynchronized and synchronized UT2 cells (1×10^6 cells/flask) were switched to 10% FCS/mevalonate-free medium. The number of surviving cells was determined every 24 h using the dye exclusion (trypan blue) method. The results represent the average (□, synchronized cells; ■, asynchronized cells) of three experiments performed in duplicate, ± the standard error. HMG-CoA reductase activity in synchronized UT2 cells was 23 ± 4 pmol min^{-1} mg^{-1} compared to 6 ± 3 pmol min^{-1} mg^{-1} in the asynchronized UT2 cell population.

The UT2* cells derived from the synchronized population of UT2 cells were single cell cloned and were utilized in the all of the experiments reported in this study. In addition, we also verified that these cells have the same two point mutations in the ER HMG-CoA reductase gene as previously reported for the UT2* cells obtained from asynchronized UT2 cells (*18*).

Determination of the HMG-CoA Reductase Activity in CHO, UT2, and UT2 Cell Extracts.* HMG-CoA reductase activity levels of UT2* cells derived from synchronized UT2 cells were similar to activity levels observed previously in UT2* cells obtained from asynchronized UT2 cells (*16*). HMG-CoA reductase activity was 275 pmol min^{-1} mg^{-1} in normal CHO cells and 6 and 52 pmol min^{-1} mg^{-1} for UT2 and UT2* cells, respectively (data not shown).

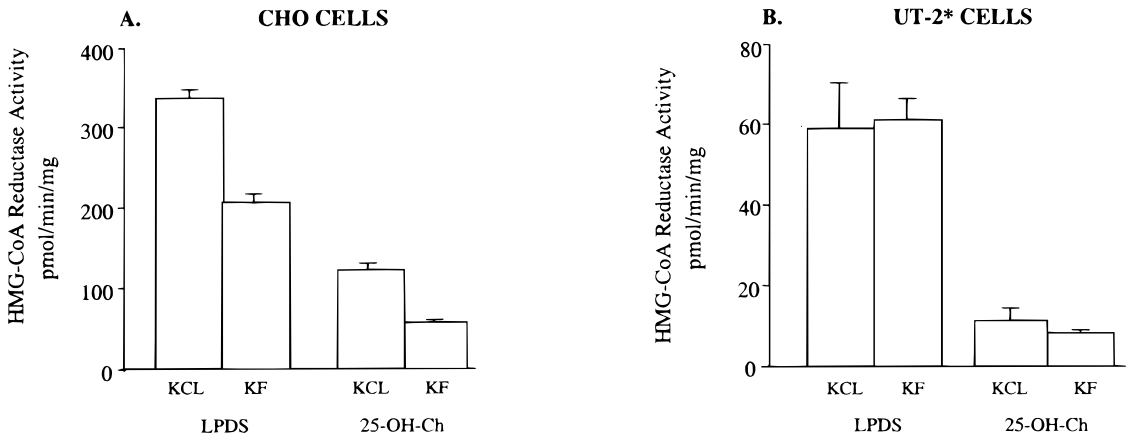

FIGURE 2: Determination of HMG-CoA reductase activity in CHO and UT2* cells in the presence of KCL and KF. Cells were cultured by standard methods in either 5% LPDS-supplemented medium or LPDS-supplemented medium containing 2 mg/mL 25-hydroxycholesterol for 16 h. The cells were washed and harvested, and HMG-CoA reductase activity was determined in the presence of 50 mM KCL or 50 mM KF. Each value represents the average of three experiments performed in duplicate, ± the standard error.

Rate of Cholesterol Biosynthesis in CHO, UT2, and UT2 Cells As Measured by the Level of Incorporation of [^{3}H]-Mevalonic Acid.* Even though the UT2 cells and CHO cells have vastly different levels of HMG-CoA reductase activity, the rate of cholesterol biosynthesis from mevalonate was shown to be very similar in these two cell lines (*17*). To determine if this was also true for the UT2* cells, we measured the rate of incorporation of [^{3}H]mevalonic acid into cholesterol in the CHO, UT2, and UT2* cells. No significant difference in the rates of incorporation of [^{3}H]-mevalonic acid into cholesterol was observed among the three cell lines (data not shown). This is in agreement with previous studies (*17*) and suggests that the isoprenoid biosynthetic pathway after the HMG-CoA reductase step is unaltered and similar in all three cell lines.

Is the Peroxisomal HMG-CoA Reductase Activity Regulated by Phosphorylation? The ER HMG-CoA reductase activity is modulated by reversible phosphorylation on Ser 871 of the reductase protein (*22*). When the ER reductase protein is phosphorylated, it is catalytically inactive. The phosphorylation process is catalyzed by an ATP-dependent kinase which is activated when the cellular levels of AMP are elevated (*25*, *26*). To determine if the peroxisomal reductase activity is regulated similarly by a phosphorylation−dephosphorylation mechanism, we determined total HMG-CoA reductase activity in CHO and UT2* cell extracts after harvesting the cells in the presence of 50 mM KCL or 50 mM KF after growth in medium containing LPDS or supplemented with 25-hydroxycholesterol (Figure 2). As expected, the HMG-CoA reductase activity measured in CHO cell extracts (Figure 2A) decreased when CHO cells were harvested in the presence of 50 mM KF, an inhibitor of cellular phosphatases. In UT2* cells, the reductase activity remained unchanged in the presence of KF (Figure 2B). However, UT2* cells grown in medium supplemented with 25-hydroxycholesterol demonstrated a significant decrease in HMG-CoA reductase activity, compared to cells grown in LPDS-containing medium. Thus, total reductase activity decreased in both cell lines in response to 25-hydroxycholesterol, but only CHO cell reductase activity exhibited a marked decrease in the presence of KF, suggesting that the peroxisomal reductase activity may not be regulated by phosphorylation and dephosphorylation.

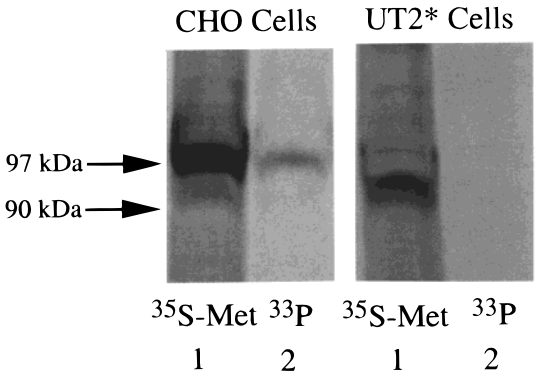

FIGURE 3: Immunoprecipitation of ^{35}S- and ^{33}P-radiolabeled HMG-CoA reductase in CHO and UT2* cells. Cells were incubated in 5% LPDS-supplemented medium for 18 h and then radiolabeled with either trans [^{35}S]methionine (lane 1) or [^{33}P]orthophosphate (lane 2) for 1 or 2 h, respectively. Following incubation, cells were rinsed with either methionine/cysteine-free medium or phosphate-free medium and harvested in the presence of phosphatase inhibitors, and the detergent-solubilized cell extracts were immunoprecipitated with anti-HMG-CoA reductase IgG and resolved by SDS−PAGE. The radiolabeled proteins were visualized with a Molecular Dynamics PhosphorImager.

To determine if the peroxisomal HMG-CoA reductase protein is phosphorylated, CHO and UT2* cells were radiolabeled with either [^{35}S]methionine or [^{33}P]phosphate (Figure 3). The radiolabeled proteins were extracted and immunoprecipitated using anti-HMG-CoA reductase antisera. In CHO cells radiolabeled with [^{35}S]methionine (lane 1) or [^{33}P]phosphate (lane 2), a 97 kDa protein was immunoprecipitated. In UT2* cells, after radiolabeling with [^{35}S]-methionine (lane 1), a 90 kDa protein is immunoprecipitated; however, after labeling with [^{33}P]phosphate (lane 2), no detectable radiolabeled band is observed. These data suggest that unlike the ER reductase, the peroxisomal reductase neither is a phosphorylated protein nor is regulated by phosphorylation and dephosphorylation, since no incorporation of [^{33}P]phosphate was observed in the 90 kDa reductase protein and the reductase activity was not modulated by using inhibitors of cellular phosphatases.

Effect of Depleting ATP Levels on Cholesterol Biosynthesis in CHO and UT2 Cells.* Previous studies have demonstrated that the phosphorylation and dephosphorylation of the ER HMG-CoA reductase regulate the levels of cholesterol

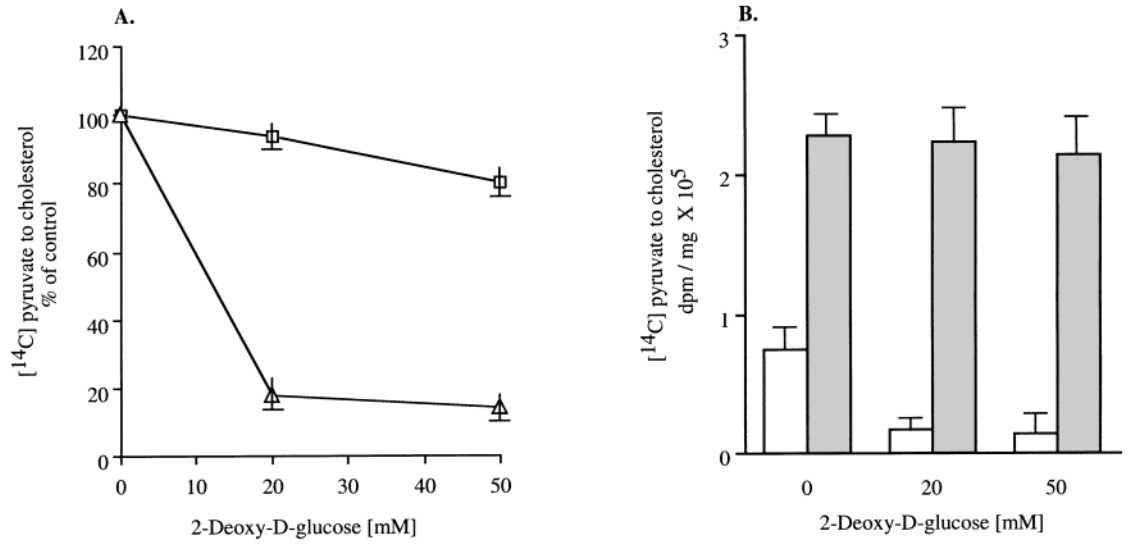

FIGURE 4: Effect of 2-deoxy-D-glucose on the rate of cholesterol biosynthesis in CHO and UT2* cells as measured by the level of incorporation of [^{14}C]pyruvate. CHO and UT2* cells were cultured in 5% LPDS-containing medium for 24 h. The cells were washed with PBS and incubated as described in Experimental Procedures in the presence of 20 mM 2-deoxy-D-glucose and 50 mM 2-deoxy-D-glucose. Cells were radiolabeled for 1 h with 3.8 μCi of [^{14}C]pyruvate sodium salt (2.8×10^3 dpm nmol^{-1} flask^{-1}, 1.5 mM). (A) Rate of incorporation of [^{14}C]pyruvate into cholesterol expressed as a percentage of the control: CHO cells (△) and UT2* cells (□). (B) Rate of cholesterol biosynthesis expressed as disintegrations per minute per milligram of cell protein for CHO (white bars) and UT2* cells (gray bars). Each value represents the average of three experiments performed in duplicate, ± the standard error.

biosynthesis in response to cellular ATP levels (*22*). Cholesterol biosynthesis is suppressed when cellular ATP levels are depleted, and AMP levels are elevated. This regulation is mediated by an AMP-dependent kinase which phosphorylates the ER reductase and decreases its activity which then downregulates sterol biosynthesis (*22*). As expected, in CHO cells treated with increasing concentrations of 2-deoxy-D-glucose (an inhibitor of glycolysis), the level of cholesterol biosynthesis was decreased, as measured by the level of incorporation of [^{14}C]pyruvate (Figure 4A). In contrast, 2-deoxy-D-glucose had very little effect on the rate of cholesterol biosynthesis in UT2* cells (Figure 4A). Taken together, these data are consistent with the conclusion that the peroxisomal reductase is not regulated by phosphorylation.

Unexpectedly, however, the rate of incorporation of [^{14}C]-pyruvate into cholesterol was 2−3-fold higher in UT2* cells than in CHO cells (Figure 4B). These results were surprising due to the fact that in CHO cells HMG-CoA reductase activity was at least 5-fold higher than in UT2* cells. Thus, to determine if this phenomenon was unique to pyruvate, we also tested the rate of incorporation of [^{3}H]acetate into cholesterol in CHO, UT2*, and UT2 cells (Figure 5). Utilizing [^{3}H]acetate as the substrate yielded results in CHO and UT2* cells similar to those observed with [^{14}C]pyruvate (Figure 4B). The rate of cholesterol biosynthesis in UT2* cells was again 2−3 times higher than that observed in CHO cells. The rate of cholesterol biosynthesis in UT2 cells (cells grown in the presence of mevalonate) was significantly lower than in CHO cells, and in agreement with previous studies (*17*).

We next tested the effects of acetate concentration and time of incubation on the rate of cholesterol biosynthesis in CHO and UT2* cells. The rate of incorporation of [^{3}H]acetate into cholesterol remained 2−3-fold higher in UT2* cells than in CHO cells at acetate concentrations of 0.5−5 mM as well as with different incubation times (data not shown).

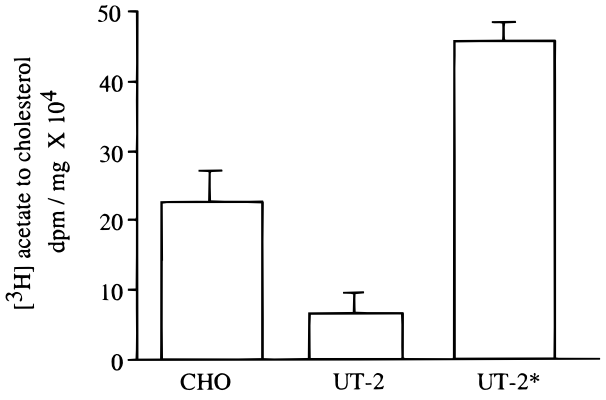

FIGURE 5: Rate of cholesterol biosynthesis in CHO, UT2, and UT2* cells as determined by the level of incorporation of [^{3}H]acetate. CHO and UT2* cells were cultured in 5% LPDS-containing medium for 24 h. UT2 cells were grown in 5% FCS medium supplemented with 0.2 mM mevalonate. Cells were radiolabeled for 4 h with 5 μCi of [^{3}H]acetate (1.1×10^5 dpm nmol^{-1} flask^{-1}). The rate of cholesterol biosynthesis is expressed as disintegrations per minute per milligram of cell protein. Each value represents the average of five experiments performed in duplicate, ± the standard error.

Rate of Cholesterol Biosynthesis in CHO and UT2 Cells As Measured by the Level of Incorporation of ^3H_2O and [^{14}C]-Acetate.* As an additional control, to verify that indeed the rate of cholesterol biosynthesis is higher in UT2* cells than in CHO cells, we also incubated the cells with ^3H_2O and [^{14}C]acetate. Again, the rate of cholesterol biosynthesis was higher in UT2* cells, when incubated in the presence of ^3H_2O and [^{14}C]acetate, and the ratios of ^{14}C to ^{3}H were consistent between the two cell lines (data not shown). Taken together, these data indicate that although HMG-CoA reductase activity levels are lower in UT2* cells, than in CHO cells, clearly the rate of cholesterol biosynthesis, as determined by three different substrates, is greater in UT2* cells.

Rate of HMG-CoA Biosynthesis in CHO, UT2, and UT2 Cells As Measured by the Level of Incorporation of [^{3}H]-*

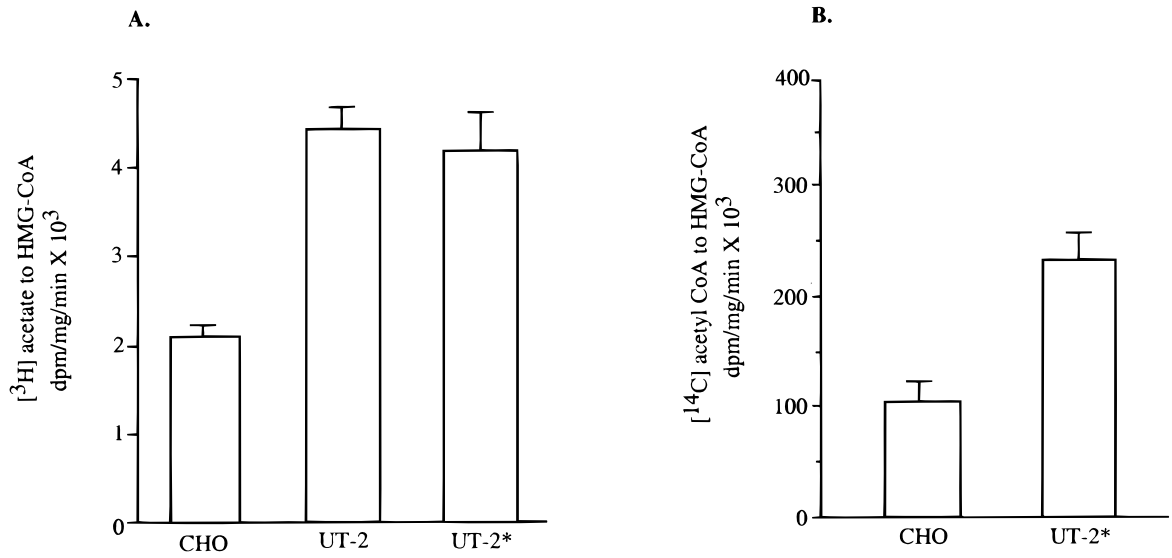

FIGURE 6: Rate of HMG-CoA biosynthesis in vivo and in vitro in CHO and UT2* cells as determined by the level of incorporation of [^{3}H]acetate and [^{14}C]acetyl CoA into HMG-CoA. CHO, UT2, and UT2* cells were maintained in 5% LPDS-containing medium supplemented with 5 μM lovastatin and 0.2 mM mevalonate, 16 h prior to the experiment. (A) Cells were radiolabeled with 5 μCi of [^{3}H]acetate (1.1×10^5 dpm nmol^{-1} flask^{-1}) for 1 h. (B) Cell homogenates were incubated with [^{14}C]acetyl CoA (1 μCi/mL) for 1 h. The rate of HMG-CoA production was then determined as described in Experimental Procedures. Each value represents the average of three experiments performed in duplicate, ± the standard error.

Table 1: Activities of HMG-CoA Synthase and HMG-CoA Reductase and the Rate of Sterol Biosynthesis in CHO and UT2* Cells in the Presence of an HMG-CoA Synthase Inhibitor[a]

	CHO cells			UT2* cells		
[L-659,699] (μM)	HMG-CoA synthase activity (nmol min^{-1} mg^{-1})	HMG-CoA reductase activity (pmol min^{-1} mg^{-1})	rate of sterol biosynthesis of [^{3}H]acetate to cholesterol (dpm/mg)	HMG-CoA synthase activity (nmol min^{-1} mg^{-1})	HMG-CoA reductase activity (pmol min^{-1} mg^{-1})	rate of sterol biosynthesis of [^{3}H]acetate to cholesterol (dpm/mg)
0	0.67 ± 0.09	230 ± 12	171000	2.09 ± 0.07	53 ± 3	414000
1	0.31 ± 0.10	476 ± 31	71500 (42%)	1.25 ± 0.13	80 ± 11	20000 (5%)
3	0.24 ± 0.08	ND	26000 (15%)	0.65 ± 0.04	ND	12000 (3%)

[a] Cells were incubated in the presence or absence of the indicated concentrations of the HMG-CoA synthase inhibitor for 3 h, and the enzyme activities and extents of cholesterol biosynthesis determined as described in Experimental Procedures. Values are the average of three experiments performed in duplicate, ± the standard error. Values in parentheses represent the rate of cholesterol biosynthesis expressed as a percentage of the control.

Acetate. To explain the higher rates of cholesterol biosynthesis observed in UT2* cells when utilizing substrates requiring the HMG-CoA reductase step, we next investigated the cholesterol biosynthesis pathway prior to the HMG-CoA reductase step. We determined the rate of HMG-CoA biosynthesis in CHO, UT2, and UT2* cells utilizing [^{3}H]-acetate as the substrate. These experiments were performed in the presence of 5 μM lovastatin to inhibit the formation of any products downstream of HMG-CoA reductase (Figure 6A). The results show that the rate of HMG-CoA biosynthesis was 2−3-fold higher in UT2 and UT2* cells than in CHO cells. In addition, we also determined the rate of conversion of [^{14}C]acetyl CoA to HMG-CoA in CHO and UT2* cells in vitro (Figure 6B). These results confirmed that there is an increased level of production of HMG-CoA in UT2* cells.

Activity Levels of Acetoacetyl CoA Thiolase and HMG-CoA Synthase in CHO and UT2 Cells.* To determine if the increased levels of HMG-CoA were due to changes in the activity levels of acetoacetyl CoA thiolase or HMG-CoA synthase, the activities of these enzymes were measured in both cell lines. The activity of acetoacetyl CoA thiolase was similar in both cell lines (data not shown); however, the activity of HMG-CoA synthase was 2−3-fold higher in UT2* cells than in CHO cells (Table 1). A similar increase in HMG-CoA synthase activity has been previously reported in UT2 cells (*17*).

Activities of HMG-CoA Synthase and HMG-CoA Reductase and the Rate of Cholesterol Biosynthesis in CHO and UT2 Cells in the Presence of an HMG-CoA Synthase Inhibitor.* To evaluate the possibility that the difference in the rate of incorporation of acetyl CoA into sterol in the two cell lines was due to the elevated levels of HMG-CoA synthase activity in the UT2* cells, we determined the activity of HMG-CoA synthase and HMG-CoA reductase and the rate of sterol biosynthesis after incubating CHO and UT2* cells in the presence and absence of a specific inhibitor of HMG-CoA synthase, L-659,699 (Table 1). The β-lactone, L-659,699 inhibitor, acylates HMG-CoA synthase in a reaction which is irreversible in vitro, but easily reversed in cultured cells (*27*, *28*). Table 1 illustrates that HMG-CoA synthase activity was inhibited by the β-lactone to similar levels in CHO and UT2* cells and that HMG-CoA reductase activity was increased in both cell lines in the presence of the β-lactone. However, the rate of cholesterol biosynthesis was 42% of the control rate in the presence of 1 μM

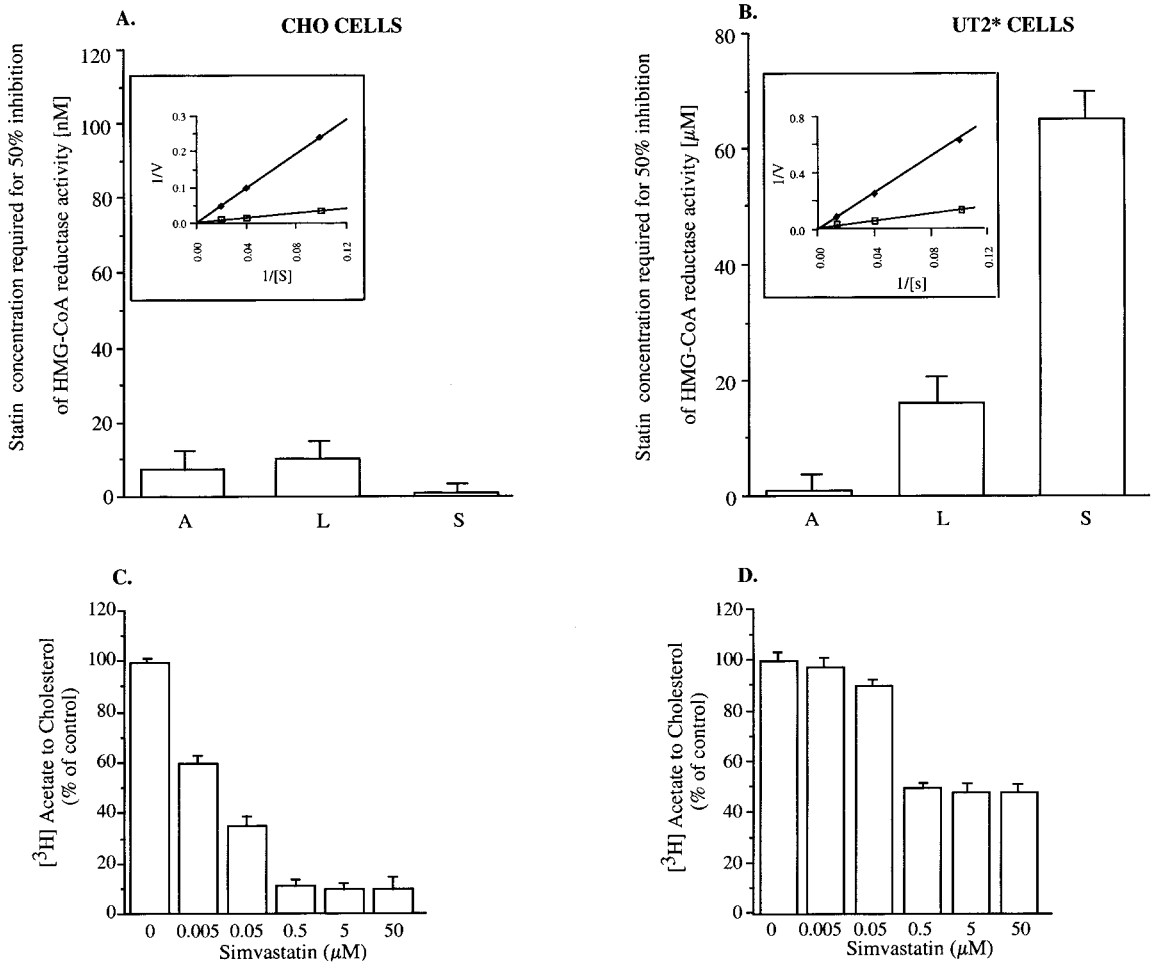

FIGURE 7: In vitro inhibition of HMG-CoA reductase activity in the presence of statins and the in vivo effects of simvastatin on cholesterol biosynthesis in CHO and UT2* cells. CHO and UT2* cells were maintained in 5% LPDS-containing medium overnight. Panels A and B illustrate the statin concentration required to reduce HMG-CoA reductase activity in vitro to 50% of control levels. The statin concentrations were obtained from concentration curves generated for each statin. Each value represents the average of three experiments performed in duplicate, ± the standard error. The statins that are used are (A) atorvastatin, (L) lovastatin, and (S) simvastatin. The insets of panels A and B show Lineweaver−Burk plots of increasing substrate concentrations in the presence (□) or absence (◆) of lovastatin. For CHO cells, a final lovastatin concentration of 1 nM was used, and for UT2* cells, that concentration was 1 μM. In panels C and D, CHO and UT2* cell media were supplemented with the indicated concentrations of simvastatin for 30 min. Cells were then radiolabeled with 5 μCi of [^{3}H]-acetate (1.1×10^5 dpm nmol^{-1} flask^{-1}) for 3.5 h. Each value represents the average of three experiments performed in duplicate, ± the standard error.

β-lactone in CHO cells, and less than 5% of the control rate in UT2* cells. These data suggest that perturbation of HMG-CoA synthase activity in UT2* cells has a dramatic effect on the carbon flux and that HMG-CoA synthase, and not HMG-CoA reductase, may be the rate-limiting enzyme for sterol biosynthesis in these cells.

Sensitivity of Peroxisomal HMG-CoA Reductase to Inhibition by Statins. Statins are known to act as potent competitive inhibitors of the ER HMG-CoA reductase (*29*−*31*). We first determined in both cell lines the in vitro IC_{50} values for three statins (atorvastatin, lovastatin, and simvastatin) (Figure 7A,B). Only nanomolar concentrations of these inhibitors were required to achieve 50% inhibition of reductase activity in CHO cells, whereas micromolar concentrations were necessary to achieve the same degree of inhibition in UT2* cells. We also evaluated if the mechanism of inhibition of the peroxisomal HMG-CoA reductase by the statins was similar to that of the ER reductase. HMG-CoA reductase activity was determined with increasing concentrations of substrate, in the presence or absence of a constant concentration of lovastatin present in the reaction mixture. The results are presented as a Lineweaver−Burk plot of enzyme kinetics (insets of panels A and B of Figure 7). These data suggest that in UT2* cells, the statins behave as competitive inhibitors of the peroxisomal reductase and indicate that the catalytic active site of the peroxisomal reductase may be structurally different from that of the ER reductase, since a 1000-fold greater concentration of statins is required for the inhibition of the peroxisomal reductase.

Effect of Simvastatin on the in Vivo Rate of Cholesterol Biosynthesis in CHO and UT2 Cells.* We next investigated if the same concentrations of the statins were required in vivo to inhibit sterol biosynthesis that were observed in vitro for inhibition of reductase activity. Since simvastatin exhibited the greatest IC_{50} difference between the CHO and UT2* cells (Figure 7A,B), we determined the concentration of simvastatin required for 50% inhibition of cholesterol biosynthesis in vivo (Figure 7C,D). The results show that 5 nM simvastatin was sufficient to decrease the rate of cholesterol biosynthesis in CHO cells to approximately 55% of the control rate, whereas at least 500 nM simvastatin was required to achieve an equivalent effect in UT2* cells. These

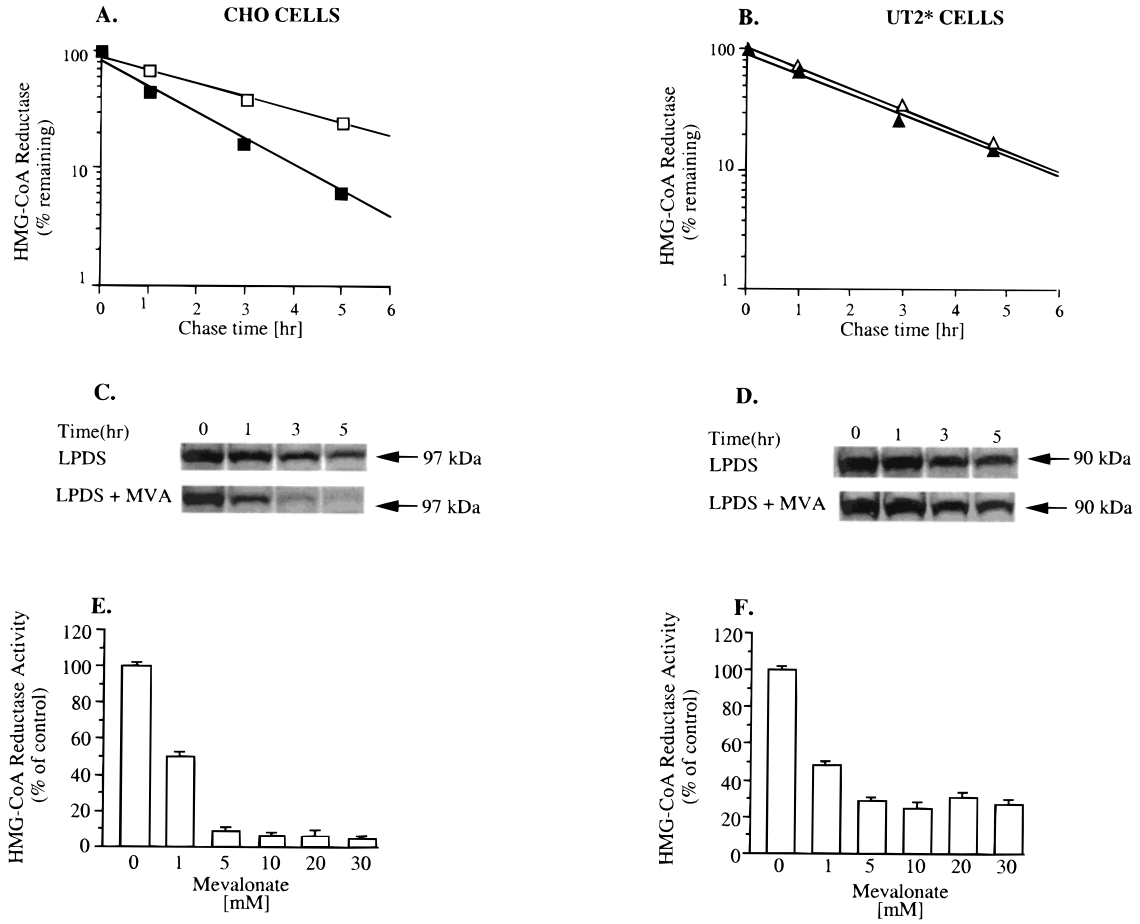

FIGURE 8: Mevalonate enhances degradation of HMG-CoA reductase in CHO cells but not in UT2* cells. As described in Experimental Procedures, CHO and UT2* cells were pulse-labeled, and then the chase was done in the presence (■) or absence (□) of 20 mM mevalonate for 0, 1, 3, and 5 h. Reductase was immunoprecipitated and subjected to SDS−PAGE. ^{35}S-labeled proteins were visualized with a Molecular Dynamics PhosphorImager and quantified. Panels A and B are a compilation of four experiments, and panels C and D illustrate the image from a representative experiment. In panels E and F, CHO and UT2* cells grown in 5% LPDS-supplemented medium were incubated with increasing concentrations of mevalonate for 3 h. Whole cell extracts were then assayed for HMG-CoA reductase activity. Each value represents the average of eight or nine experiments performed in duplicate, ± the standard error.

results support the in vitro data (Figure 7A,B) and indicate that the relative resistance of the peroxisomal reductase to statins is also evident in vivo.

Does Mevalonate Affect the Rate of Degradation of HMG-CoA Reductase in UT2 Cells?* It is well-known that mevalonate (*32*−*34*) and farnesol (*35*, *36*) accelerate the rate of degradation of the ER HMG-CoA reductase protein. To determine if the same mode of regulation controls the peroxisomal reductase, we determined the half-life of HMG-CoA reductase in CHO and UT2* cells in the presence and absence of sodium mevalonate in a pulse−chase radiolabeling protocol (Figure 8A−D). CHO cells express an HMG-CoA reductase that is degraded relatively rapidly, with a half-life of approximately 3.5 h. Supplementation of CHO cells with sodium mevalonate results in a significant acceleration of the degradation rate (1.4 h) (Figure 8A,C), consistent with previous reports (*37*). In contrast, the HMG-CoA reductase half-life in UT2* cells is approximately 5 h, and the addition of sodium mevalonate does not influence this rate of degradation (5.3 h) (Figure 8B,D). When UT2* cells are supplemented with farnesol, again, no change in the rate of degradation of HMG-CoA reductase is observed (data not shown).

Effect of Mevalonate on HMG-CoA Reductase Activity in CHO and UT2 Cells.* We also evaluated the effect of mevalonate on HMG-CoA reductase activity in CHO and UT2* cells. Cells were treated for 3 h with increasing concentrations of sodium mevalonate, and HMG-CoA reductase activity was determined (Figure 8E,F). In CHO cells, reductase activity decreased nearly to 50% of control values when the mevalonate concentration approached 1 mM, and the activity was suppressed to approximately 3% of control values with mevalonate concentrations above 5 mM (Figure 8E). In UT2* cells, reductase activity decreased to 50% of control values at a similar mevalonate concentration of 1 mM; however, at mevalonate concentrations of ≥5 mM, the reductase activity remained at approximately 30% of the control values (Figure 8F). These results indicate that UT2* cell HMG-CoA reductase activity is more resistant to mevalonate than CHO cell HMG-CoA reductase activity.

Does ALLN Influence the Rate of Degradation of UT2 Cell HMG-CoA Reductase?* The cysteine protease inhibitor, ALLN, is known to inhibit the degradation of ER HMG-CoA reductase, both in vitro and in vivo (*38*, *39*). To investigate the effects of ALLN on UT2* cells, the cells were supplemented with either ALLN, sodium mevalonate, or ALLN with sodium mevalonate, and the cell extracts were immunoblotted for HMG-CoA reductase (Figure 9). As illustrated, the presence of ALLN had a dramatic effect on increasing the levels of the CHO cells' ER reductase protein

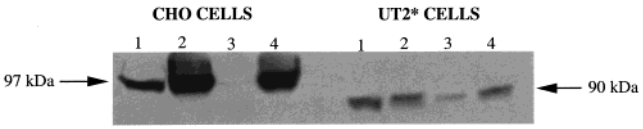

FIGURE 9: HMG-CoA reductase degradation is blocked by ALLN in CHO cells but not in UT2* cells. CHO and UT2* cells were maintained in 5% LPDS-containing medium supplemented with 5 μM lovastatin and 0.2 mM mevalonate. The cells were then supplemented with either ALLN (50 μg/mL), sodium mevalonate (20 mM), or ALLN (50 μg/mL) with sodium mevalonate (20 mM) and incubated for 20 h, and the cell extracts (200 μg) were immunoblotted for HMG-CoA reductase activity: lane 1, no additions; lane 2, ALLN; lane 3, mevalonate; and lane 4, ALLN with mevalonate.

over control levels (Figure 9, right panel, lane 2), whereas there was no increase over control levels in the reductase protein in UT2* cells in the presence of ALLN (Figure 9, left panel, lane 2). As expected, when CHO cells were supplemented with mevalonate, there was a significant decrease in the reductase protein levels, as compared to the control lane (Figure 9, right panel, lane 3). In UT2* cells, in the presence of mevalonate, there is also a reduction of the reductase protein level (Figure 9, left panel, lane 3), however, to a much lesser degree than that observed in CHO cells. These data are consistent with the previous observation that the reductase activity in UT2* cells was also more resistant to mevalonate downregulation than the reductase activity in CHO cells (Figure 8E,F).

DISCUSSION

In this study, we utilized the UT2* cells as a model system to characterize the 90 kDa peroxisomal HMG-CoA reductase independent of the ER reductase. We made the unexpected observation that the rate of incorporation of either pyruvate, acetate, or ^3H_2O into cholesterol was 2−3-fold higher in UT2* cells than in CHO cells (Figures 4 and 5). These results contradicted the HMG-CoA reductase activity measurements showing that the reductase activity of CHO cells was 4−5-fold higher than the reductase activity of UT2* cells (Table 1). The discrepancy in these results was not due to different acetate pool concentrations of the two cell lines or the lack of linearity in the rate of incorporation of substrate into cholesterol. In addition, since all three substrates produced similar results, the higher rate of cholesterol biosynthesis observed in the UT2* cells could not be the result of a higher rate of conversion of acetate to acetyl CoA in the UT2* cells. Furthermore, we also ruled out the possibility that we were underestimating the reductase activity measurements in UT2* cells.

Thus, to explain the higher rates of cholesterol biosynthesis observed in UT2* cells when utilizing substrates requiring the HMG-CoA reductase step, we next investigated the cholesterol biosynthetic pathway prior to HMG-CoA reductase. The results demonstrated an increased rate of HMG-CoA production in UT2* cells compared to that in CHO cells, which was due to elevated levels of HMG-CoA synthase activity (Table 1), and not acetoacetyl CoA thiolase. Enhanced HMG-CoA synthase activity levels have been reported previously in UT2 cells (*17*).

A number of earlier reports have described a similar dissociation between HMG-CoA reductase activity levels and the rate of sterol biosynthesis in a variety of cultured cells (*40*−*42*). The results obtained from these studies implied that under certain conditions, HMG-CoA synthase may be the rate-limiting enzyme of sterol biosynthesis instead of HMG-CoA reductase (*40*−*42*). To determine if HMG-CoA synthase could be the rate-limiting enzyme for sterol biosynthesis in the UT2* cells, we inhibited HMG-CoA synthase activity with a specific HMG-CoA synthase inhibitor (the β-lactone, L-659,699) (*27*, *28*). HMG-CoA synthase activity was inhibited by the β-lactone to similar levels in both CHO and UT2* cells. This level of inhibition produced a dramatic decline in the rate of sterol biosynthesis in UT2* cells, while in contrast, the rate of cholesterol biosynthesis in CHO cells was much less affected (Table 1). These results suggest that unlike in CHO cells, the rate of sterol biosynthesis in UT2* cells may be coordinately regulated with HMG-CoA synthase activity and that the peroxisomal HMG-CoA reductase may not be regulating this pathway. These results also demonstrate that although the peroxisomal reductase produces sufficient levels of mevalonate for cell growth and can compensate for the absence of the ER HMG-CoA reductase in UT2* cells, it does not serve the same function in the regulation of cholesterol biosynthesis.

We have also demonstrated a number of other major differences between the two reductases. Our data indicate that in UT2* cells the peroxisomal HMG-CoA reductase protein is not phosphorylated, the activity of the reductase is not influenced by phosphatase inhibitors, and the rate of cholesterol biosynthesis is not affected by cellular ATP levels (Figures 2−4). These results suggest that either the peroxisomal reductase protein lacks a phosphorylation site or the peroxisomal localization prevents the access of a kinase activity. However, previous studies have shown that peroxisomal proteins can be phosphorylated (*43*, *44*); therefore, it is unlikely that the peroxisomal reductase protein would be masked from the activity of kinases.

In mammalian cells, both sterol and non-sterol products of mevalonate are required for full suppression of ER reductase activity. Sterols are assumed to suppress transcription of the reductase gene via an octanucleotide sequence in the promoter region (*45*). Non-sterol metabolites appear to inhibit translation of reductase mRNA (*10*, *11*), and both sterols and early pathway products (non-sterols) enhance the degradation of the ER HMG-CoA reductase protein (*11*, *37*). ALLN (an inhibitor of cysteine proteases and the proteasome) has also been shown to inhibit the effect of mevalonate degradation of the ER HMG-CoA reductase (*38*, *39*). As expected, in CHO cells the addition of mevalonate accelerated the degradation of HMG-CoA reductase and decreased the reductase activity to 3% of control levels, and the addition of ALLN inhibited the in vivo basal and mevalonate-accelerated degradation of the ER reductase (Figures 8 and 9). In contrast, the addition of mevalonate to the UT2* cells had no effect on the degradation of the peroxisomal reductase (Figure 8B,D); reductase activity levels were reduced to 30% of the control level (Figure 8F), and the addition of ALLN did not influence the turnover of the protein (Figure 9).

The accelerated degradation of the ER HMG-CoA reductase protein is dependent upon the transmembrane region of the protein; however, the entire membrane domain of the ER HMG-CoA reductase is not required for the protein's regulated degradation (*46*−*50*). Therefore, the differences observed in the stability of the two proteins in response to

mevalonate could imply that the specific determinants required for the regulated degradation of the ER protein are not present in the peroxisomal protein, that the peroxisomal reductase contains other determinants that program its stability, or that the conformation of the peroxisomal reductase prevents its degradation.

Another difference observed between the two enzymes is in their relative sensitivity to inhibition by statins. The peroxisomal reductase was shown to be 1000 times more resistant to inhibition than the reductase activity determined in CHO cells (Figure 7). The same results were observed with three different statins, suggesting that the conformation of the active site might be different in the two enzymes. It is significant to note that previous studies in cultured cells have reported the requirement of low concentrations of statins (nanomolar) for inhibition of sterol biosynthesis, but micromolar concentrations for inhibition of isoprenylation of proteins (*23*, *51*). One interpretation of these results could be that the ER reductase is first inhibited (low concentrations) and the higher concentration requirement is for inhibiting the peroxisomal reductase, therefore, suggesting a role for the peroxisomal reductase in non-sterol metabolite production. This hypothesis is also supported by the observation that the peroxisomal reductase activity is induced at the G_0/G_1 interphase of the cell cycle in synchronized UT2 cells (Figure 1). This observation suggests that a product of mevalonate might be required at this stage of the cell cycle.

From these studies, it is clear that the peroxisomal enzyme in these cells is sufficient to allow cell survival in the absence of mevalonate. This is also true in yeast, where either isozyme Hmg1p or Hmg2p can fulfill the essential requirement for HMG-CoA reductase activity (*52*). The levels of both yeast isozymes increase in response to drugs or genetic manipulations which decrease mevalonate levels; however, the mechanism(s) of this regulation is different for each isozyme (*53*−*55*).

The results of our experiments demonstrate a striking similarity between the peroxisomal reductase and the yeast Hmg1p. Both proteins are not regulated by phosphorylation (*56*). They are also very stable proteins in response to isoprenoids that have been shown to accelerate the degradation of the ER reductase in CHO cells and Hmg2 in yeast. Furthermore, both proteins do not appear to require the proteosomal apparatus for degradation since their degradation is not blocked by ALLN.

In summary, we have shown that the HMG-CoA reductase activities in CHO and UT2* cells exhibit similar responses to downregulation by 25-hydroxycholesterol and mevalonate. Although, the reductase activity in UT2* cells is more resistant to downregulation by mevalonate. However, a number of significant differences were observed between the two proteins. The peroxisomal reductase is not the rate-limiting enzyme for cholesterol biosynthesis. The peroxisomal protein is not phosphorylated. The reductase activity in UT2* cells is 1000-fold more resistant to inhibition by statins. Mevalonate does not alter the rate of degradation of the peroxisomal protein. Finally, the degradation of the protein is not affected by ALLN. Taken together, the data support the conclusion that in UT2* cells the HMG-CoA reductase is localized to peroxisomes, is structurally different from the ER HMG-CoA reductase, and plays a different role in isoprenoid metabolism and regulation.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1980) *J. Lipid Res. 21*, 505−517.
2. Keller, G. A., Barton, M. C., Shapiro, D. J., and Singer, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 770−774.
3. Keller, G. A., Pazirandeh, M., and Krisans, S. (1986) *J. Cell Biol. 103*, 875−886.
4. Krisans, S. K. (1996) *Ann. N.Y. Acad. Sci. 804*, 142−164.
5. Appelkvist, E. L., and Kalen, A. (1989) *Eur. J. Biochem. 185*, 503−509.
6. Liscum, L., Finer-Moore, J., Stroud, R. M., Luskey, K. L., Brown, M. S., and Goldstein, J. L. (1985) *J. Biol. Chem. 260*, 522−530.
7. Goldstein, J. L., and Brown, M. S. (1990) *Nature 343*, 425−430.
8. Liscum, L., Luskey, K. L., Chin, D. J., Ho, Y. K., Brown, M. S., and Goldstein, J. L. (1983) *J. Biol. Chem. 258*, 8450−8455.
9. Osborne, T. F., Goldstein, J. L., and Brown, M. S. (1985) *Cell 42*, 203−212.
10. Peffley, D., and Sinensky, M. (1985) *J. Biol. Chem. 260*, 9949−9952.
11. Nakanishi, M., Goldstein, J. L., and Brown, M. S. (1988) *J. Biol. Chem. 263*, 8929−8937.
12. Kumagi, H., Chun, K. T., and Simoni, R. D. (1995) *J. Biol. Chem. 270*, 19107−19113.
13. McGee, T. P., Cheng, H. H., Kumagi, H., Omura, S., and Simoni, R. D. (1996) *J. Biol. Chem. 271*, 25630−25638.
14. Ericsson, J., and Edwards, P. A. (1998) *J. Biol. Chem. 273*, 17865−17870.
15. Brown, M. S., and Goldstein, J. L. (1997) *Cell 89*, 331−340.
16. Engfelt, W. H., Shackelford, J. E., Aboushadi, N., Jessani, N., Masuda, K., Paton, V. G., Keller, G. A., and Krisans, S. K. (1997) *J. Biol. Chem. 272*, 24579−24587.
17. Mosley, S. T., Brown, M. S., Anderson, R. G. W., and Goldstein, J. L. (1983) *J. Biol. Chem. 258*, 13875−13881.
18. Engfelt, W. H., Masuda, K., Paton, V. G., and Krisans, S. K. (1998) *J. Lipid Res. 21*, 505−517.
19. Balasubramaniam, S., Goldstein, J. L., and Brown, M. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1421−1425.
20. Clinkenbread, K. D., Sugiyama, T., Moss, J., Reed, W. D., and Lane, M. D. (1973) *J. Biol. Chem. 248*, 2275−2284.
21. Aboushadi, N., and Krisans, S. K. (1998) *J. Lipid Res. 39*, 1781−1789.
22. Sato, R., Goldstein, J. L., and Brown, M. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9261−9265.
23. Maltese, W. A. (1990) *FASEB J. 4*, 3319−3328.
24. Sepp-Lorenzo, L., Rao, S., and Coleman, P. S. (1991) *Eur. J. Biochem. 200*, 579−590.
25. Hardie, D. G. (1992) *Biochim. Biophys. Acta 1123*, 231−238.
26. Hardie, D. G., and MacKintosh, R. W. (1992) *BioEssays 14*, 699−704.
27. Greenspan, M. D., Bull, H. G., Yudkovitz, J. B., Hanf, D. B., and Alberts, A. W. (1993) *Biochem. J. 289*, 889−895.
28. Greenspan, M. D., Yudkovitz, J. B., Lo, C. Y., Chen, J. S., Alberts, A. W., Hunt, V. M., Chang, M. N., Yang, S. S., Thompson, K. L., and Chiang, Y. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 488−492.
29. Slater, E. E., and MacDonald, J. S. (1988) *Drugs 36*, 72−82.
30. Stone, B. G., Evans, C. D., Prigge, W. F., Duane, W. C., and Gebhard, R. L. (1989) *J. Lipid Res. 30*, 1943−1952.
31. Ngata, Y., Hidaka, Y., Ishida, F., and Kamei, T. (1990) *Biochem. Pharmacol. 40*, 843−850.
32. Chin, D. J., Gil, G., Faust, J. R., Goldstein, J. L., Brown, M. S., and Luskey, K. L. (1985) *Mol. Cell. Biol. 5*, 634−641.
33. Straka, M. S., and Panini, S. R. (1995) *Arch. Biochem. Biophys. 317*, 235−243.
34. Peffley, D. M. (1992) *Somatic Cell Mol. Genet. 18*, 19−32.
35. Correll, C. C., Ng, L., and Edwards, P. A. (1994) *J. Biol. Chem 269*, 17390−17393.
36. Meigs, T. E., Roseman, D. S., and Simoni, R. D. (1996) *J. Biol. Chem. 271*, 7196−7922.
37. Roitelman, J., and Simoni, R. D. (1992) *J. Biol. Chem. 267*, 25264−25273.

38. Inoue, S., Bar-Nun, S., Roitelman, J., and Simoni, R. D. (1991) *J. Biol. Chem. 266*, 13311−13317.
39. Roitelman, J., Bar-Nun, S., Inoue, S., and Simoni, R. D. (1991) *J. Biol. Chem. 266*, 16085−16091.
40. Volpe, J. J., and Obert, K. A. (1981) *Arch. Biochem. Biophys. 212*, 88−97.
41. Cavenee, W. K., and Melnykovych, G. (1978) *J. Biol. Chem. 252*, 3272−3276.
42. Johnston, D., Cavenee, W. K., Ramachandran, C. K., and Melnkovych, G. (1980) *Biochim. Biophys. Acta 572*, 188−192.
43. Skorin, C., Soto, U., Necochea, C., and Leighton, F. (1986) *Biochem. Biophys. Res. Commun. 15*, 188−194.
44. Elgersma, Y., Kwast, L., Van den berg, M., Snyder, W. B., Distel, B., Subramani, S., and Tabak, H. F. (1997) *EMBO J. 16*, 7326−7341.
45. Osborne, T. F. (1991) *J. Biol. Chem. 266*, 13947−13951.
46. Gil, G., Faust, J. R., Chin, D. J., Goldstein, J. L., and Brown, M. S. (1985) *Cell 41*, 249−258.
47. Skalnik, D. G., Narita, H., Kent, C., and Simoni, R. D. (1988) *J. Biol. Chem. 263*, 6863−6841.
48. Olender, E. H., and Simoni, R. D. (1992) *J. Biol. Chem. 267*, 4223−4235.
49. Jingami, H., Brown, M. S., Goldstein, J. L., Anderson, R. G. W., and Luskey, K. L. (1987) *J. Cell Biol. 104*, 1693−1704.
50. Chun, K. T., and Simoni, R. D. (1992) *J. Biol. Chem. 267*, 4236−4246.
51. Nagata, Y., Hidaka, Y., Ishida, F., and Kamei, T. (1990) *Jpn. J. Pharmacol. 54*, 315−324.
52. Basson, M. E., Thorsness, M., Finer-Moore, J., Stroud, R. M., and Rine, J. (1988) *Mol. Cell. Biol. 8*, 3797−3808.
53. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., and Darnell, J. E., Jr. (1981) *Cell 23*, 731−739.
54. Hampton, R. Y., and Rine, J. (1994) *J. Cell Biol. 125*, 299−312.
55. Dimster-Denk, D., Thorsness, M. K., and Rine, J. (1994) *Mol. Cell. Biol. 5*, 655−665.
56. Hampton, R. Y., Dimster-Denk, D., and Rine, J. (1996) *Trends Biochem. Sci. 21*, 140−145.

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