Mechanisms of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Overaccumulation in Three Compactin-resistant Cell Lines*

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We have isolated three mammalian cell lines which are resistant to compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. The drug resistance in all three cell lines is due to an increase of HMG-CoA reductase activity. Two of the three cell lines overaccumulate HMG-CoA reductase messenger RNA when grown in the presence of compactin. DNA hybridization experiments indicate that both a baby hamster kidney-derived compactin-resistant cell line, C100, and a cell line derived from mouse 3T6 cells, 3T6-40, exhibit amplifications of the HMG-CoA reductase gene. A third compactin-resistant cell line derived from Chinese hamster ovary cells, ML100, does not exhibit an amplification of the HMG-CoA reductase gene, nor does it show an elevated level of HMG-CoA reductase mRNA, comparable to that seen in the other cell lines.

3-Hydroxy-3-methylglutaryl coenzyme A reductase (mevalonate:NADP* oxidoreductase (CoA-acylating), EC 1.1.34) is the major regulatory enzyme in the cholesterol biosynthetic pathway. This pathway is also responsible for the production of isoprenoid compounds such as squalene, ubiquinone and dolichol. Hence, the efficient regulation of HMG-CoA reductase activity is essential for a wide range of cellular functions. Addition of low density lipoprotein to the growth medium has been shown to decrease HMG-CoA reductase activity in tissue culture cells (see Ref. 1 for review). The molecular interactions which allow cholesterol to reduce HMG-CoA reductase activity are still unclear. However, the regulation appears to predominantly involve modulation of mRNA levels (2-4).

One of the major difficulties in studying the regulation of this system is that HMG-CoA reductase normally comprises only a small fraction of the cell's protein, perhaps 0.01% when fully induced (5). The discovery of compactin (ML236B), a competitive inhibitor of HMG-CoA reductase, by Endo and co-workers (6) overcame this difficulty. We and others have selected compactin-resistant cell lines which overaccumulate HMG-CoA reductase (7-9). In these cells, HMG-CoA reductase comprises as much as 1-2% of the total protein of the cell.

In this paper we have used an HMG-CoA reductase cDNA clone to study the mechanisms utilized to overaccumulate HMG-CoA reductase, and hence develop compactin resistance, in three mammalian cell lines.

EXPERIMENTAL PROCEDURES

Materials—All materials, unless specified, were readily available from commercial sources. All restriction endonucleases and nick translation components were obtained from Bethesda Research Laboratories and nitrocellulose filters were obtained from Schleicher & Schuell. Antibody directed against rat liver HMG-CoA reductase was a generous gift of Hans-Stephan Jenke, Gesellschaft fur Strahlen und Umweltforschung Institut fur Zellchemie, Munich, Federal Republic of Germany. Compactin was the generous gift of A. Endo, Department of Agricultural and Biological Chemistry, Tokyo Noko University, Tokyo, Japan, and was converted from the lactone to the acid form as described (7).

Selection and Growth of Compactin-resistant Cell Lines—Cells were grown in monolayers under conditions already described (7). The compactin-resistant mouse cell line was selected by treating 3T6 cells with increasing concentrations of compactin. At each step of compactin treatment there was greater than 90% cell death. Cells growing in 5% fetal calf serum were treated with 5, 10, 20, and 40 μg/ml of compactin. A single colony was isolated after the last compactin treatment and denoted 3T6-40. This cell line was maintained in delipidated media (7) containing 40 μg/ml of compactin for the duration of the experiments described here. The original selection of a precursor of the CHO-derived compactin-resistant cell line described here has previously been reported (7). This cell line, ML8, was further selected for resistance to 40 and 100 μg/ml of compactin. The selection of the BHK-derived compactin-resistant cell line, C100, is described elsewhere (8). Both the C100 and ML100 cell lines were maintained in delipidated media containing 50 μg/ml of compactin during the experiments described here.

Determination of HMG-CoA Reductase Activity—Cell extracts were prepared as described (7) and assayed for HMG-CoA reductase activity by the method of Shapiro et al. (10). 35 nmol of HMG-CoA (13,500 dpm/nmol) was included in each incubation. 50-100 μg of protein from extracts of parental and compactin-resistant cells grown in the absence of compactin, or 0.5-1.0 μg of protein from compactin-resistant cells grown in the presence of compactin, were assayed. All assays were performed in duplicate, and HMG-CoA reductase activity was linear with respect to protein concentration as determined by the method of Lowry et al. (11).

Immunoprecipitation of HMG-CoA Reductase—Cells were incubated on day 0 in 25-cm² flasks at a density of 0.5 × 10⁶ cells/flask and were fed on day 2. On day 3 media was removed and replaced with 1 ml of labeling media. This consisted of 0.9 ml of minimal essential medium prepared without methionine with 5% dialyzed delipidated fetal calf serum, plus 0.1 ml of minimal essential medium...
with 5% delipidated fetal calf serum, prepared as usual. Cells were labeled for 24 h with 50 μCi/ml of [35S]methionine. Extracts were prepared, and 100 μg of protein was immunoprecipitated as described (8). Samples were analyzed by 10% acrylamide/sodium dodecyl sulfate gel electrophoresis as described by Laemmli (12). Gels were fluorographed by the method of Laskey (13).

Isolation of a cDNA Clone of HMG-CoA Reductase—We utilized pRED10, a derivative of pBR322 containing 1.2 kilobase pairs of HMG-CoA reductase cDNA (generously provided by Joseph Goldstein, Michael Brown, Daniel Chin, and Kenneth Luskey, Department of Molecular Genetics, University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, TX), to screen a AGT10 library. This library was prepared with cDNA synthesized from mRNA isolated from our ClOO compactin-resistant cell line using techniques described elsewhere (14–17). Plaque hybridizations were carried out as described by Davis et al. (18). 40 pRED10 positive clones were isolated. We chose λDS11, which contains approximately 4 kilobase pairs of HMG-CoA reductase cDNA, to use as a probe to determine the HMG-CoA reductase gene copy number and mRNA levels in our compactin-resistant cell lines.

**RESULTS**

Levels of HMG-CoA Reductase—When all three compactin-resistant cell lines are grown in the presence of compactin the specific activity of HMG-CoA reductase rises roughly 50- to 100-fold relative to the parental levels (Table I). These values must be considered approximations because of uncertainty and variability in measuring the low HMG-CoA reductase activity in parental cells. It is clear, however, that these cell lines are resistant to compactin because they have increased HMG-CoA reductase activity. We have previously demonstrated by immunoprecipitations that the increased activity in the C100 cell line is due to an overaccumulation of HMG-CoA reductase (8). This is also the case for the 3T6-40 and ML100 cell lines as demonstrated in Fig. 1 (lanes 1).

We have examined the ability of these cells to regulate the activity of HMG-CoA reductase (Table I). When C100 or ML100 cells were grown in the absence of compactin for a period of 10–14 days the activity of HMG-CoA reductase returned to parental values. However, HMG-CoA reductase activity in similarly treated 3T6-40 cells remains about 10-fold elevated over the parental level. The reason for this difference between the cell lines is unclear. The reduction in activity after compactin removal is paralleled by a reduction in the amount of enzyme present as determined by immunoprecipitation (Fig. 1, lanes 3) (8).

**HMG-CoA Reductase mRNA Levels in Compactin-resistant Cell Lines**—We performed RNA filter hybridization experiments, using λDS11 (HMG-CoA reductase) as a probe, to quantitate the relative amount of RNA in various samples. Multiple exposures were scanned to confirm that each band was within the linear response range of the film.

**Table I**

| Cell type | Reductase activity | Reductase mRNA levels | Reductase mRNA levels in compactin resistant cells relative to parental cells |
|-----------|--------------------|-----------------------|--------------------------------------------------------------------------------|
| ClOO      | 0.08–0.4           | 0.2                   | ND^4                                                                           |
| C100 OFF  | 0.15–0.2           | 0.7                   | 3.5–5.9                                                                        |
| C100 ON^2 | 6–10               | 2.4                   | 12–13                                                                          |
| C100 ON^* | 12–15              | ND^4                  |                                                                                |
| 3T6^6     | 0.08–0.1           | 0.4                   | 1                                                                              |
| 3T6-40 OFF | 0.6–1.0            | 0.6                   | 1.2–1.5                                                                        |
| 3T6-40 ON^* | 10–11              | 2.1                   | 5.3–10.8                                                                      |
| CHO^6     | 0.2–0.4            | 0.3                   | 1                                                                              |
| ML100 OFF^v | 0.3–0.9            | 0.2                   | 1.2–1.5                                                                        |
| ML100 ON^v | 8–15               | 0.2                   | 1.2–1.5                                                                        |

^* Ranges represent a minimum of 4 determinations.

^b Ranges represent a summary of both Northern analysis and hybridization of HMG-CoA reductase or α-tubulin clones to cytoplasmic RNA applied directly to nitrocellulose filters (slot blots).

^c Cells grown in the absence of compactin for 10–14 days.

^d Cells grown in the presence of 50 μg/ml of compactin.

^e Cells grown in the presence of 100 μg/ml of compactin.

^f ND, not determined.

^g Cells grown in the presence of 40 μg/ml of compactin.
determine if our compactin-resistant cell lines had elevated levels of HMG-CoA reductase mRNA. The results of a typical Northern blot experiment, shown in Fig. 2, indicate HMG-CoA reductase mRNA overaccumulates in the C100 and 3T6-40 cell lines when each is grown in the presence of compactin. Quantitation of the HMG-CoA reductase mRNA levels (Table I) in the C100 and 3T6-40 cells from several such experiments, using pT1 (α-tubulin) to normalize the values, indicates that the levels of mRNA in these two cell lines are equivalent when grown in the presence of compactin. In addition to using α-tubulin as a control, similar results were obtained using pR12, a dihydrofolate reductase cDNA clone, to normalize the values. Although the absolute amount of HMG-CoA reductase mRNA in the 3T6-40 line is comparable to that found in the C100 line, the -fold increase appears smaller due to an apparently higher level of HMG-CoA reductase mRNA in the parental mouse line (3T6). The quantitation of mRNA levels in parental cells must be considered approximate, however, due to the difficulty of detecting the low level of HMG-CoA reductase mRNA in these cells. Hence, it may be misleading to present these results in terms of -fold increase. In addition to Northern analysis, similar results have been obtained by binding total cytoplasmic RNA directly to nitrocellulose.

It is clear that the HMG-CoA reductase mRNA levels decrease upon compactin removal in both the C100 and 3T6-40 lines (Table I and Fig. 2, lanes 2). In the C100 cells, however, the mRNA level remains significantly elevated over the parental level. At the same time, the specific activity of HMG-CoA reductase has dropped to the parental value (Table I). Decreased translational efficiency, decreased enzyme activity due to allosteric or covalent regulation, or increased enzyme degradation could account for this result. This last explanation seems likely since we have previously demonstrated (4) a 6-fold increase in the degradation rate of HMG-CoA reductase in the C100 cell line in response to the removal of compactin from the growth medium. Conversely, the level of HMG-CoA reductase mRNA in the 3T6-40 cell line drops to the parental level when these cells are grown in the absence of compactin for 10–14 days while HMG-CoA reductase activity remains 10-fold elevated (Table I). We do not understand the mechanism behind this phenomenon and it again may reflect the poor quantitation of low levels of HMG-CoA reductase activity and mRNA.

Most surprising was the finding that the level of HMG-CoA reductase mRNA is not elevated in the ML100 cell line to the extent seen in the other cell lines (Fig. 2, CHO panel), even though these cells have approximately a 50-fold elevation of HMG-CoA reductase (Table I), comparable to that seen in the C100 and 3T6-40 cell lines. We do not yet understand how the ML100 cell line achieves this overaccumulation of HMG-CoA reductase.

**HMG-CoA Reductase Gene Number**—One mechanism which would allow cells to overaccumulate HMG-CoA reductase mRNA would be an increase in the number of copies of the corresponding gene. To test for this possibility we determined the HMG-CoA reductase gene copy number by DNA filter hybridization experiments. We probed HindIII-digested DNA isolated from our compactin-resistant cell lines with λDS11 (HMG-CoA reductase).

The mouse line, 3T6-40, exhibits a 10-fold gene amplification which is lost when these cells are grown in the absence of compactin for approximately 150 generations (Fig. 3, 3T6 panel). This is consistent with H-33258 fluorescence visualized double minute chromosomes seen in these cells (data not shown). The double minute chromosomes disappear coincidentally with the loss of HMG-CoA reductase sequences as indicated by the filter hybridization experiments.

The BHK-derived compactin-resistant cell line, C100, has a stable 5-fold amplification of the HMG-CoA reductase gene consistent with the amplified sequences existing on a chromosome (Fig. 3, BHK panel). These cells have been grown for nine months in the absence of compactin and were found to retain their resistant phenotype.

There is no apparent gene amplification in the ML100 cell line (Fig. 3, CHO panel). This was expected since the level of
HMG-CoA reductase mRNA is not elevated in these cells to the extent seen in the other cell lines. The mechanism which allows these cells to contain elevated levels of HMG-CoA reductase is unknown. It is important to realize that the regulatory alteration in these cells does not lead to a constitutive production of HMG-CoA reductase. The specific activity of HMG-CoA reductase and immunoprecipitable protein drops when compactin is removed from the media (Table I and Fig. 1).

**DISCUSSION**

We report the isolation and characterization of three mammalian cell lines which are resistant to compactin, a competitive inhibitor of HMG-CoA reductase. By performing immunoprecipitations (Fig. 1) and measuring the specific activity of HMG-CoA reductase (Table I) we have shown that all three cell lines overcome the toxicity of compactin by an overaccumulation of the target enzyme.

It has previously been reported (23) that the compactin-resistant cell line UT-1 contains a 100-fold elevation in both HMG-CoA reductase activity and mRNA when grown in the presence of compactin. While the C100 and 3T6-40 cell lines described here exhibit approximately a 50- to 100-fold elevation in HMG-CoA reductase activity when grown in the presence of compactin, they do not appear to exhibit a corresponding elevation in HMG-CoA reductase mRNA levels. Both Northern analysis (Fig. 2) and hybridization of ADS11 to total cytoplasmic RNA applied directly to nitrocellulose filters (data not shown) indicate a 5- to 13-fold elevation in HMG-CoA reductase mRNA levels. Our estimates for -fold elevation are not precise since it is difficult to accurately measure HMG-CoA reductase mRNA levels in parental cells. However, it seems likely that the C100 and 3T6-40 cell lines may additionally enhance translation of HMG-CoA reductase mRNA.

The nature of the mutation in the ML100 cell line is not yet known. Among the alterations which could contribute to the observed phenotype are an increase in the translation of HMG-CoA reductase mRNA or a decrease in the degradation rate of the enzyme. Further study of this cell line is needed to identify the nature of the alteration in the HMG-CoA reductase regulatory machinery.

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