Multivalent Control of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase

MEVALONATE-DERIVED PRODUCT INHIBITS TRANSLATION OF mRNA AND ACCELERATES DEGRADATION OF ENZYME*

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The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) is subject to multivalent feedback suppression mediated by sterols and non-sterol substances derived from mevalonate, the product of the enzyme. To dissect the mechanism for this multivalent effect, Chinese hamster ovary cells were incubated with sterols contained in plasma lipoproteins and with a high concentration (100μM) of compactin, an inhibitor of the reductase. Under these conditions, the amounts of HMG-CoA reductase protein and catalytic activity were high, although the cells were saturated with sterols, as reflected by active synthesis of cholesteryl esters. The amount of enzyme fell by 90% when the cells received excess mevalonate in addition to sterols. This decline was not associated with a fall in levels of reductase messenger RNA (mRNA). Rather, it was attributable to an 80% decline in translation of the mRNA, coupled with a 5-fold increase in the rate of degradation of reductase protein, as revealed by pulse-chase experiments with [35S]methionine. Considered together with previous data, these findings suggest a multilevel mechanism for multivalent regulation of HMG-CoA reductase. We suggest that sterols suppress the enzyme incompletely by partially repressing transcription of the gene and that nonsterol products derived from mevalonate further reduce the enzyme by inhibiting translation of the mRNA. Sterols and non-sterol products, acting together, accelerate the degradation of reductase protein. This combination of transcriptional and posttranslational controls can regulate the amount of reductase protein over a several hundred-fold range in animal cells.

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) catalyzes the synthesis of mevalonate, a crucial intermediate in the formation of sterols and non-sterol isoprenoid compounds. The activity of this enzyme in animal cells is subject to multivalent control (reviewed in Ref. 1). Full suppression requires the presence of sterols plus another product derived from mevalonate, apparently a non-sterol metabolite such as dolichol, ubiquinone, or isopentenyl tRNA. Under the usual conditions employed to study regulation of HMG-CoA reductase in tissue culture, only the sterol-dependent component is observed. Thus, when tissue culture cells are grown in the absence of exogenous cholesterol, the amount of HMG-CoA reductase activity is high. Enzyme activity is suppressed by 75–90% when exogenous sterols are present, either in the form of cholesterol contained in plasma low density lipoprotein or in the form of hydroxylated sterols such as 25-hydroxycholesterol, which are added to the culture medium in solvents. Even in the presence of maximal levels of sterols, however, repression of the enzyme is never complete. The cells always retain sufficient HMG-CoA reductase to produce small amounts of mevalonate that are preferentially shunted into the non-sterol products (1).

The non-sterol component of this regulatory mechanism is unmasked when the cells are incubated with compactin or other related inhibitors of HMG-CoA reductase (1). Under these conditions the cells can no longer form the mevalonate needed for non-sterol products, and the amount of enzyme decreases, even in the presence of sterols. It can be suppressed only when exogenous mevalonate is supplied.

The mechanisms for this multivalent feedback regulation are turning out to be quite complex. One well-established mechanism involves repression of transcription of the HMG-CoA reductase gene by sterols (2–4). This repression is dependent upon a short regulatory sequence in the 5′-flanking region of the gene. When this sequence is altered, HMG-CoA reductase transcription becomes constitutive and there is no suppression by sterols (4).

Another mechanism for regulation is controlled degradation of the enzyme, an event that is dependent upon its association with the endoplasmic reticulum membrane (6–8). HMG-CoA reductase is a protein with a molecular weight of 97,000 which is divided into two regions (9). The NH2-terminal third of the protein is extremely hydrophobic and is firmly embedded in the membranes of the endoplasmic reticulum. The COOH-terminal two-thirds of the protein, which is hydrophilic, contains all of the catalytic activity. Altered cDNAs encoding deleted forms of HMG-CoA reductase have been introduced into Chinese hamster ovary (CHO) cells by transfection (8, 10). When the cDNA encodes the entire enzyme, the protein is attached to the endoplasmic reticulum, and its degradation is accelerated by sterols. On the other hand, when the cDNA
encodes a truncated form of the reductase that lacks the membrane spanning regions, the enzyme is soluble. This cysteic enzyme has an extremely long half-life, and its degradation is not accelerated by sterols (8). The studies summarized above lead to the conclusion that sterols suppress HMG-CoA reductase by at least two mechanisms: 1) decreased transcription of the gene; and 2) enhanced degradation of the enzyme.

In contrast to the knowledge about mechanisms of sterol-mediated suppression, the regulation by nonsterol mevalonate-derived products is much less understood. In an important study, Peffley and Sinensky (11) showed that the addition of mevalonate in the presence of sterols inhibits the translation of HMG-CoA reductase mRNA in a line of CHO cells that is deprived of mevalonate as a result of a genetic defect in HMG-CoA synthase, the enzyme preceding HMG-CoA reductase in the cholesterol biosynthetic pathway.

In the current studies we have sought to explore the mechanism for the sterol-independent suppression of HMG-CoA reductase. For this purpose we have developed a new protocol using CHO cells that have never been adapted to growth in the presence of compactin. These cells are incubated acutely with a high level of compactin, thereby abolishing mevalonate production. They are also incubated with sterols contained in plasma lipoproteins or with oxygen-derived products is much less understood. In an important study, Peffley and Sinensky (11) showed that the addition of mevalonate in the presence of sterols inhibits the translation of HMG-CoA reductase mRNA in a line of CHO cells that is deprived of mevalonate as a result of a genetic defect in HMG-CoA synthase, the enzyme preceding HMG-CoA reductase in the cholesterol biosynthetic pathway.

IN EXPERIMENTAL PROCEDURES

Materials—DL-Mevalonic acid lactone was purchased from Fluka Chemical Corp. and converted to the sodium form (1 M stock solution) as described (12). Compactin in the lactone form was kindly provided by Akira Endo (Tokyo Noko University, Tokyo, Japan) and converted to the sodium form (10 mM stock solution) as described (12). We obtained [35S]methionine (100 Ci/mmol) from ICN Radiochemicals; prelabeled molecular weight markers from Sigma; Immun-Blot assay kit from BioRad Laboratories; Pansorbin. Immunoblot analysis of HMG-CoA reductase protein was carried out using the Bio-Rad Immun-Blot assay kit as described (22).

Other Assays—Immunoprecipitation of [35S]methionine-labeled cell extracts was carried out as described (10) except that protein A-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) was used instead of Pansorbin. Immunoblot analysis of HMG-CoA reductase protein was carried out using the Bio-Rad Immun-Blot assay kit as described (10) except that immunoprecipitation of the polyclonal rabbit antibody was used instead of nitrocellulose and a monoclonal antibody, respectively. Molecular mass standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis contained bovine serum albumin (68,000 Da), myoglobin (16,000 Da), ovalbumin (42,000 Da), carbonic anhydrase (30,000 Da), RNA polymerase (300,000 Da), and phosphoglycerate kinase (100,000 Da). The incorporation of [3H]oleate into cholesteryl [3H]oleate by cell monolayers was determined as described (22).

RESULTS

To study the effects of exogenous mevalonate on HMG-CoA reductase, it was necessary to completely inhibit mevalonate production in vivo with high levels of compactin (100 µM). In preliminary experiments we found that these high concentrations of compactin persisted in the detergent-solubilized extracts and inhibited reductase activity when assayed in vitro. To overcome this problem, we subjected the extract to extensive dialysis. Fig. 1 shows a validation experiment in which we added a low concentration of compactin (5 µM) directly to a detergent-solubilized cell extract in vitro. This level of compactin completely inhibited HMG-CoA reductase activity in the extract (zero time, Fig. 1). We then dialyzed the extract in either small-pore membranes (6,000–8,000 dalton cutoff, open circles) or large-pore membranes (12,000–14,000-dalton cutoff, open triangles). At various intervals aliquots were removed and saved for assay of reductase activity. As a control, we also dialyzed samples that had not received compactin (closed circle and triangle). The inhibition by com-
Fig. 1. Removal of compactin by dialysis. Detergent-solubilized extracts were prepared from CHO cells that were grown for 24 h in LPDS as described under "Experimental Procedures." Compacting (5 μM) was added to the extracts and were incubated for 20 min at 37 °C. The extracts (in a volume of 200 μl) were then dialyzed at 4 °C against 200 ml of buffer B at 4 °C in a membrane with either a 6,000–8,000-dalton cut-off (C) or a 12,000–14,000-dalton cut-off (A). During dialysis, 20-μl aliquots from each extract were removed at the indicated time and kept at 4 °C until the end of the experiment when all assays of HMG-CoA reductase were performed. The 100% of control activity denotes the reductase activity of non-dialyzed samples containing no compactin and kept at 4 °C for 51 h (1 nmol·min⁻¹·mg protein⁻¹). Closed symbols (●, ○) indicate samples that did not receive compactin but were subjected to dialysis for 51 h in the small-pore (●) and large-pore (○) membranes. Arrows denote the times when buffer was changed.

Fig. 2. Growth of CHO cells in the presence of compactin and various concentrations of mevalonate. On day 0, cells were seeded at 700 cells/well in a 6-well culture plate in 2 ml of medium A containing 10% NCS. On day 2, the cells received 2 ml of medium A supplemented with 10% NCS, 100 μM compactin where indicated, and the indicated concentration of mevalonate. The medium was changed every day. On day 9, the cells were fixed and stained with crystal violet.

compactin was reversed more rapidly with the large-pore membranes than with the small-pore membranes. In both cases, by 51 h the compactin inhibition had been totally removed. At this point the activity in the dialyzed samples was actually somewhat greater than was observed in undialyzed extracts in the absence of compactin (designated 100% of control). The activity was linear with increasing amounts of extract in the assay (data not shown). For the remainder of the experiments in this study, we used the small-pore membranes and dialyzed the extracts for 48 h.

As would be expected from previous data (1, 23), compactin at 100 μM prevented long term growth of CHO cells even though their cholesterol requirements were being satisfied by the lipoproteins present in NCS (Fig. 2). Long term growth was restored when the cells were cultured with 0.2 mM mevalonate, an amount that satisfied the cells' requirement for non-cholesterol, mevalonate-derived substances.

Fig. 3 shows the short term effects of mevalonate on HMG-CoA reductase activity of CHO cells incubated for 24 h under various conditions. When CHO cells were incubated in LPDS

Fig. 3. Regulation of HMG-CoA reductase activity by mevalonate in CHO cells. Cells were set up for experiments on day 0 in medium A containing 10% NCS. On day 1, the cells were switched to medium A supplemented with 10% NCS, 100 μM compactin, and the indicated concentration of mevalonate (Panel A) or with 10% LPDS or 10% NCS in the absence of compactin (Panel B). After incubation for 24 h, cells were harvested for measurement of HMG-CoA reductase activity. Circles and triangles denote two separate experiments. Closed symbols denote cells that received exogenous sterols (10 μg/ml cholesterol plus 0.5 μg/ml 25-hydroxycholesterol) on day 1. Each value represents the average of duplicate incubations.

and in the absence of compactin, HMG-CoA reductase activity was in the range of 2,200 pmol·min⁻¹·mg protein⁻¹ (Fig. 3B). In the presence of 10% NCS, the activity was suppressed by nearly 90% to the range of 300 pmol·min⁻¹·mg protein⁻¹ (Fig. 3B). The addition of compactin plus a low level of mevalonate (0.01–0.2 mM) in the presence of 10% NCS caused HMG-CoA reductase to rise by 10-fold to 2500 pmol·min⁻¹·mg protein⁻¹ (Fig. 3A). Increasing the concentration of mevalonate above 0.2 mM led to a >95% reduction in reductase activity (Fig. 3A). Thus, compactin elevates reductase activity even in the presence of NCS, and this elevation can be reversed by the inclusion of high levels of mevalonate in the culture medium.

To exclude the possibility that the number of LDL receptors was insufficient to supply a saturating amount of sterol to the cell in the presence of NCS, we added a high level of 25-hydroxycholesterol (0.5 μg/ml) plus cholesterol (10 μg/ml). This concentration of sterols is 10 times higher than the concentration needed to suppress HMG-CoA reductase activity in the absence of serum lipoproteins and compactin (3). However, in the presence of compactin and 10% NCS these sterols did not further suppress HMG-CoA reductase activity (closed circle and triangle in Fig. 3A).

The changes in HMG-CoA reductase activity in Fig. 3 were associated with changes in the amount of HMG-CoA reductase protein as determined by immunoblotting (Fig. 4). When cells were grown in the presence of LPDS, the amount of HMG-CoA reductase protein was high (lane 1), and it was strongly reduced when NCS was present (lane 2). When we added compactin plus a low level of mevalonate (0.1 mM) in the presence of NCS, the amount of HMG-CoA reductase increased (lane 3). The reductase protein began to decrease when the mevalonate concentration approached 0.4 mM (lane 6), and the amount of enzyme protein was strongly reduced at mevalonate concentrations of 10–20 mM (lanes 10 and 11). As an additional control in this experiment, we measured the amount of HMG-CoA reductase protein in cells that were
we measured the rate of incorporation of [14C]oleate into high this excess of sterols, the amount of reductase protein was grown in the presence of LPDS, and it was stimulated nearly cholesterol [14C]oleate formation was low when cells were pulse-labeled for 2 h with 0.1 mM [14C]oleate-albumin (1,000 cpm/nmol), after which the cells were harvested to determine their content of cholesteryl [14C]oleate (Table I). This reaction, which is mediated by acyl-coenzyme A:cholesterol acyltransferase (24), is stimulated when the intracellular level of cholesterol exceeds metabolic requirements. As shown in Table I, the rate of cholesterol [14C]oleate formation was low when cells were grown in the presence of LPDS, and it was stimulated nearly 10-fold by NCS. The NCS also suppressed HMG-CoA reductase activity. When 100 μM compactin and 0.2 mM mevalonate were present, cholesterol esterification remained high, indicating that the cells were continuing to obtain abundant sterols. Yet under these conditions, HMG-CoA reductase activity rose by nearly 10-fold. When the concentration of mevalonate was raised to 20 mM, reductase activity was suppressed by nearly 99%, but there was only a slight further increase in the rate of cholesteryl [14C]oleate formation. To further confirm that NCS was supplying sufficient sterol to cells in the presence of 100 μM compactin, we compared the amounts of HMG-CoA reductase and HMG-CoA synthase protein by immunoblotting. HMG-CoA synthase, the enzyme that precedes the reductase in the cholesterol synthetic pathway, is also negatively regulated by lipoprotein-derived cholesterol (13). As shown in Fig. 5, when cells were grown in LPDS, the amounts of reductase and synthase protein were both high. When NCS was substituted for LPDS, the amount of reductase and synthase were both dramatically reduced (lane 2). The addition of compactin plus 0.2 mM mevalonate markedly increased the amount of HMG-CoA reductase protein, but the amount of HMG-CoA synthase protein remained suppressed (lane 3). When the concentration of mevalonate was raised to 20 mM, HMG-CoA reductase protein was markedly reduced (lane 4). The data of Table I and Fig. 5 confirm that in the presence of 100 μM compactin, NCS was capable of supplying sufficient sterol to activate cholesteryl ester synthesis and to repress HMG-CoA synthase, but that HMG-CoA reductase remained elevated unless high levels of mevalonate were also present. Mevalonate repressed HMG-CoA reductase activity in part because it inhibited synthesis of the enzyme. Fig. 6 shows an experiment in which cells were incubated with [35S]methionine for 25 min, and the incorporation into HMG-CoA reductase was measured by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography. In LPDS the rate of synthesis of the enzyme was high (lane 1), and it was markedly reduced when NCS was added (lane 2). In NCS plus compactin and low mevalonate, there was a slight, but significant increase in the rate of enzyme synthesis (lane 3). In the presence of 20 mM mevalonate, synthesis of HMG-CoA reductase was nearly abolished (lane 4).

To determine whether the changes in synthetic rates of

![Fig. 4](image-url)

**Fig. 4. Immunoblot analysis of HMG-CoA reductase in CHO cells.** Cells were set up for experiments on day 0 and fed on day 1 as described in the legend to Fig. 4. Additions to the culture medium were indicated at the bottom. 10% LPDS, 10% NCS, 100 μM compactin, 0.5 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol [sterols], or the indicated concentration of mevalonate. Cells were harvested on day 2. Aliquots of the solubilized extracts (250 μg of protein) were added to SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon membrane, and incubated with 2 μg/ml rabbit anti-HMG-CoA reductase IgG. The membrane was then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG. The immunoreactive bands were detected by color development using reagents provided in a Bio-Rad Immun-Blot assay kit. M₅ standards are indicated.

| Addition to medium | LPDS | NCS | Compactin | Mevalonate |
|--------------------|------|-----|-----------|------------|
| %                  | μM   | mM  | molar     | pmol/min⁻¹*mg⁻¹ | pmol/h⁻¹*mg⁻¹ |
| 10                 | 10   | 0.2 | 23        | 1940        | 4540        |
| 10                 | 100  | 20  | 23        | 310         | 1830        |
| 10                 | 10   | 0.2 | 310       | 23          | 23          |
| 10                 | 100  | 20  | 310       | 1940        | 4540        |

Table I: Effect of mevalonate on HMG-CoA reductase activity and cholesteryl [14C]oleate formation in CHO cells

![Fig. 5](image-url)

**Fig. 5. Immunoblot analysis of HMG-CoA reductase and HMG-CoA synthase in CHO cells.** Cells were set up for experiments on day 0 and fed on day 1 as described in the legend to Fig. 4 with the specific additions indicated in the above table. On day 2, one set of cells was harvested for measurement of HMG-CoA reductase activity. Another set of cells were pulse-labeled for 2 h with 0.1 nM [14C]oleate-albumin (10⁶ cpm/nmol), after which the cells were harvested to determine their content of cholesteryl [14C]oleate. Each value represents the average of duplicate incubations.

1. We measured the rate of incorporation of [14C]oleate into cholesteryl [14C]oleate (Table I). This reaction, which is mediated by acyl-coenzyme A:cholesterol acyltransferase (24), is stimulated when the intracellular level of cholesterol exceeds metabolic requirements. As shown in Table I, the rate of cholesterol [14C]oleate formation was low when cells were grown in the presence of LPDS, and it was stimulated nearly 10-fold by NCS. The NCS also suppressed HMG-CoA reductase activity.

2. To further confirm that NCS was supplying sufficient sterol to cells in the presence of 100 μM compactin, we compared the amounts of HMG-CoA reductase and HMG-CoA synthase protein by immunoblotting. HMG-CoA synthase, the enzyme that precedes the reductase in the cholesterol synthetic pathway, is also negatively regulated by lipoprotein-derived cholesterol (13). As shown in Fig. 5, when cells were grown in LPDS, the amounts of reductase and synthase protein were both high. When NCS was substituted for LPDS, the amount of reductase and synthase were both dramatically reduced (lane 2). The addition of compactin plus 0.2 mM mevalonate markedly increased the amount of HMG-CoA reductase protein, but the amount of HMG-CoA synthase protein remained suppressed (lane 3). When the concentration of mevalonate was raised to 20 mM, HMG-CoA reductase protein was markedly reduced (lane 4). The data of Table I and Fig. 5 confirm that in the presence of 100 μM compactin, NCS was capable of supplying sufficient sterol to activate cholesteryl ester synthesis and to repress HMG-CoA synthase, but that HMG-CoA reductase remained elevated unless high levels of mevalonate were also present.

3. Mevalonate repressed HMG-CoA reductase activity in part because it inhibited synthesis of the enzyme. Fig. 6 shows an experiment in which cells were incubated with [35S]methionine for 25 min, and the incorporation into HMG-CoA reductase was measured by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography. In LPDS the rate of synthesis of the enzyme was high (lane 1), and it was markedly reduced when NCS was added (lane 2). In NCS plus compactin and low mevalonate, there was a slight, but significant increase in the rate of enzyme synthesis (lane 3). In the presence of 20 mM mevalonate, synthesis of HMG-CoA reductase was nearly abolished (lane 4).

4. To determine whether the changes in synthetic rates of
HMG-CoA reductase were associated with changes in the amount of reductase mRNA, we measured the amount of mRNA with a sensitive S1 nuclease protection assay using radiolabeled single-stranded cDNA probes. As a control, we also measured the amount of messenger transcribed from the gene for neomycin phosphotransferase (neo), which was contained in a permanently integrated plasmid that had been introduced into the cells by transfection (see "Experimental Procedures"). The neomycin phosphotransferase gene is driven by the SV40 promoter and its expression should not be influenced by sterols or mevalonate. When the CHO cells were grown in LPDS, the amount of reductase mRNA was high as indicated by the relatively large amount of the complementary single-stranded probe that was protected from complete digestion with the single-strand-specific S1 nuclease (reductase signal, Fig. 7, lane B). The addition of NCS markedly suppressed the amount of reductase mRNA (lane C). When compactin plus a low level of mevalonate were added, there was no significant increase in the amount of reductase mRNA (lane D). Conversely, when a high level of mevalonate (20 mM) was present, there was no reduction in the amount of HMG-CoA reductase mRNA (lane E). None of these perturbations produced a significant change in the amount of mRNA derived from the neo gene (lanes B–E).

The data of Figs. 6 and 7 suggested that the sterols in NCS suppress HMG-CoA reductase by suppressing the mRNA. On the other hand, high levels of mevalonate suppress synthesis of HMG-CoA reductase without further decreasing the amount of its mRNA. To compare these processes quantitatively, we performed densitometric scans of the autoradiograms in Figs. 6 and 7, and we also measured HMG-CoA reductase enzymatic activity in cells from the same experiment. The results are presented as Experiment 1 in Table II. For comparative purposes, we have normalized these data relative to the values obtained in cells grown in NCS without compactin (metabolic state B in Table II). The shift from NCS (state B) to LPDS (state A) produced increases in the level of reductase mRNA, in the rate of enzyme synthesis, and in HMG-CoA reductase activity. These increases ranged from 4.8- to 11.5-fold. Similar results were seen when this experiment was repeated (Experiment 2 of Table II). When the cells in NCS were treated with compactin plus a low level of mevalonate (metabolic state C), there was a slight increase in reductase mRNA and a proportional increase in the rate of enzyme synthesis, averaging approximately 1.5-fold. HMG-CoA reductase activity, however, rose by 8-fold, a rise that could not be explained by changes in the synthetic rate. When the cells were treated with a high level of mevalonate (metabolic state D), there was no significant suppression of the mRNA. However, the rate of enzyme synthesis was reduced by 80% as compared with the rate of the presence of low mevalonate (state C). The amount of HMG-CoA reductase activity was reduced even further, the activity falling by approximately 99% in the two experiments. These data indicate that the switch from low mevalonate to high mevalonate reduces HMG-CoA reductase activity by at least two mechanisms: 1) a decreased translational efficiency of the mRNA and 2) an additional effect attributable to a posttranslational mechanism.

A likely mechanism for this posttranslational regulation is enhanced degradation of the enzyme. To test this hypothesis, we performed a pulse-chase experiment in which cells were incubated with [35S]methionine and then switched to medium containing NCS and compactin with either 0.2 or 20 mM mevalonate (Fig. 8). After varying times the cells were harvested and HMG-CoA reductase was immunoprecipitated and subjected to SDS gel electrophoresis, autoradiography, and densitometry. At the low mevalonate concentration, HMG-CoA reductase protein was degraded extremely slowly. Eighty percent of the radiolabeled enzyme was still immunoprecipitable after 5 h (Fig. 8B, open triangles). On the other hand, in the presence of 20 mM mevalonate the enzyme was degraded extremely rapidly (Fig. 8B, closed triangles). After a 1-h lag phase, the enzyme disappeared with a half-life of approximately 1 h; after 5 h, less than 5% of the initial enzyme was present. Mevalonate did not have any effect on the
TABLE II
Relative effect of mevalonate on HMG-CoA reductase mRNA, translation efficiency, and enzymatic activity in CHO cells

| Addition to medium | Metabolic state | A | B | C | D |
|--------------------|----------------|---|---|---|---|
| LPDS (%)           | 10             | 10| 10| 10| 10|
| NCS (%)            | 7.1            | 1 | 1.6| 0.34|
| Compactin (μM)     | 11.5           | 1*| 8.0| 0.06|
| Mevalonate (mM)    | 5.0            | 1 | 1.3| 1.3|
|                   | 8.2            | 1 | 1.4| 0.27|
|                   | 8.7            | 1*| 6.3| 0.10|

*The absolute level of HMG-CoA reductase activity was 200 pmol·min⁻¹·mg protein⁻¹.

**The absolute level of HMG-CoA reductase activity was 270 pmol·min⁻¹·mg protein⁻¹.

catabolism of total cell protein (Fig. 8A).

Fig. 9A shows the time course of decline in HMG-CoA reductase activity after addition of 20 μM mevalonate to CHO cells incubated in NCS plus compactin. The enzyme activity fell with a half-life of 90 min, and this was associated with a parallel change in the amount of enzyme protein as determined by immunoblotting (Fig. 9A, inset). To test whether the high rate of degradation required lysosomes, we measured this degradation in the presence of chloroquine, an agent that inhibits the activity of lysosomal hydrolases by raising lysosomal pH (25) (Fig. 9B). Degradation of reductase was followed by measuring the decline in enzyme activity after new enzyme synthesis was blocked by cycloheximide. In the absence of high concentrations of mevalonate, reductase activity declined slowly after addition of cycloheximide (open circles, Fig. 9B). In the presence of high mevalonate plus cycloheximide, the enzyme declined with a half-life of 60 min (closed circles). The addition of 0.2 mM chloroquine (closed squares) did not slow this degradation. These experiments with cycloheximide confirm the conclusion from the [3H]methionine labeling studies and further indicate that mevalonate accelerates degradation of HMG-CoA reductase. They also suggest that lysosomal proteases are not involved.

In previous studies, we showed that the rapid degradation of HMG-CoA reductase was dependent on the presence of its hydrophobic NH2-terminal region, which attaches it to the membrane of the endoplasmic reticulum (8). To determine whether mevalonate-stimulated degradation also requires the membrane-spanning component, we studied this process in two lines of CHO cells that were transfected with expressible cDNAs that constitutively produce mRNA for HMG-CoA

Fig. 8. Turnover of 3H-labeled HMG-CoA reductase in CHO cells. Cells were set up for culture on day 0 in medium A supplemented with 10% NCS, 100 μM compactin, and 0.2 mM mevalonate. On day 2, cells were pulse-labeled for 4 h with 35S-methionine in medium A (3.4 μM methionine) supplemented with 10% NCS, 100 μM compactin, and 0.2 mM mevalonate. Cells were then washed once with phosphate-buffered saline, incubated with medium A containing 0.3 mM unlabeled methionine and supplemented with 10% NCS, 100 μM compactin, and either 0.2 mM (Δ) or 20 mM (A) mevalonate. After incubation for the indicated time, cells were harvested, and HMG-CoA reductase was analyzed by immunoprecipitation followed by SDS gel electrophoresis and autoradiography (see inset). Turnover of total cell protein (Panel A) was determined by trichloroacetic acid precipitation of the cellular extract and scintillation counting; 100% of control value was 2 × 10⁶ cpm. Turnover of HMG-CoA reductase (Panel B) was determined by densitometric scanning of the signal corresponding to the 97-kDa HMG-CoA reductase band shown in the inset.

Fig. 9. Mevalonate-mediated decline in HMG-CoA reductase activity in CHO cells. Cells were set up on day 0 in medium A supplemented with 10% NCS, 100 μM compactin, and 0.2 mM mevalonate and refed with the same medium on day 1. Panel A, on day 2, 20 μM mevalonate was added to the medium in a staggered fashion so that all the cells could be harvested at the same time. Panel B, on day 2, the indicated addition plus 0.5 mM cycloheximide was added to the medium in a staggered fashion so that the cells could be harvested at the same time. After incubation for the indicated time, cells were harvested, and HMG-CoA reductase was analyzed by immunoblotting (inset, Panel A) and enzyme assay. Each value represents the average of duplicate incubations. The 100% of control value for enzyme activity was 2200 pmol·min⁻¹·mg protein⁻¹.
reductase under control of the unregulated SV40 promoter (8). One cell line, designated TR-36, expresses a complete, membrane-bound HMG-CoA reductase with a molecular mass of 97 kDa. The other cell line, designated TR-70, expresses a mutant enzyme in which amino acids 10-341 have been deleted. The enzyme produced by this cDNA is fully active, has an apparent molecular mass of about 60 kDa on SDS-polyacrylamide gels, and behaves as a soluble cytosolic protein (8). In the first experiment, TR-36 cells and TR-70 cells were incubated with NCS, compactin, and varying levels of mevalonate (Fig. 10). After 24 h, the cells were harvested and HMG-CoA reductase activity was measured. In the TR-36 cells high levels of mevalonate led to a 95% reduction in HMG-CoA reductase activity (Fig. 10A). In the TR-70 cells there was no change in activity at mevalonate levels as high as 20 mM (Fig. 10B). The decline in HMG-CoA reductase activity in the TR-36 cells was associated with a disappearance of immunoreactive HMG-CoA reductase protein as determined by immunoblotting (Fig. 11). These findings suggest that the decline in enzyme activity of the TR-36 cells is due primarily to mevalonate-mediated enhancement of degradation and that this enhancement requires the attachment of the enzyme to the membranes of the endoplasmic reticulum.

**DISCUSSION**

The current data add new information to our knowledge of the multivalent regulation of HMG-CoA reductase. CHO cells develop high levels of HMG-CoA reductase protein and enzymatic activity when they are deprived of mevalonate by incubation with compactin. In the presence of sterols the mRNA is partially suppressed, but the level of reductase remains relatively high. When mevalonate is added, the mRNA does not decline further, but the level of reductase declines owing to two mechanisms: 1) a reduced translational efficiency of reductase mRNA and 2) an accelerated degradation of reductase protein.

We believe that mevalonate is regulating reductase translation and degradation either directly or through its conversion to a non-sterol substance, but not through conversion to a sterol. If mevalonate were acting through conversion to a sterol, its effects should have been reproduced when 25-hydroxycholesterol was added. Previous studies have indicated that sterols regulate HMG-CoA reductase through a common mechanism and that 25-hydroxycholesterol is among the most potent (26, 27). Since mevalonate achieves its effect even in the presence of saturating levels of 25-hydroxycholesterol and cholesterol, it is unlikely that mevalonate is acting through conversion to a sterol.

The sterol-independent effects of mevalonate are illustrated in Table II. The relevant comparison is metabolic state C (compactin plus low mevalonate) and metabolic state D (compactin plus high mevalonate). In the presence of high mevalonate, there was no significant decrease in the amount of hybridizable reductase mRNA (Table II and Fig. 7), yet there was an 80% reduction in the rate of incorporation of [35S]methionine into immunoprecipitable HMG-CoA reductase (Table II and Fig. 6). HMG-CoA reductase activity fell by 99% (Fig. 3 and Table II), a drop that was even more profound than could be explained by the 80% decline in enzyme synthetic rate. There was a corresponding fall of more than 90% in the amount of immunodetectable HMG-CoA reductase protein (Figs. 4 and 5 and Table II). These findings imply that, in addition to decreasing mRNA translation, high mevalonate concentrations increased the rate of degradation of the enzyme. This conclusion was confirmed directly by pulse-chase experiments (Fig. 8). Mevalonate increased the rate of degradation, but only when the membrane-spanning region of the reductase was present (Figs. 10 and 11). Similar results were obtained when the degradation of HMG-CoA reductase was followed by measuring the decline in enzyme activity after adding cycloheximide (Fig. 9B). Interestingly, this degradation was not inhibited by chloroquine (Fig. 9B), suggest...
ing that lysosomes may not be involved.

In previous studies we found that addition of lipoprotein-bound cholesterol or 25-hydroxycholesterol to CHO cells in the presence of compactin accelerated degradation of HMG-CoA reductase without a requirement for exogenous mevalonate (6, 8, 16). It is likely that those cells were already producing sufficient mevalonate to permit this acceleration. The cells had been adapted to grow in the presence of compactin, and therefore they were able to produce sufficient mevalonate for growth, which selected for cells that expressed levels of reductase activity that were able to partially overcome the effects of compactin. In the current studies we blocked mevalonate production completely by using cells that were never adapted to compactin and by treating them acutely with an extremely high concentration of compactin. Under these conditions the degradation of HMG-CoA reductase remained slow, even when the cells had an amount of sterols that was sufficient to repress transcription of the reductase mRNA (Fig. 8) and to maintain high rates of cholesterol esterification (Table 1). Degradation was accelerated only when high levels of exogenous mevalonate were present in addition to sterols. Since sterols accelerate degradation of HMG-CoA reductase only in the presence of mevalonate, it is possible that the sterols do not act directly, but rather through diversion of mevalonate to a non-sterol substance. When cells are grown with limiting mevalonate in the absence of sterols, most of the mevalonate is converted into sterols (reviewed in Ref. 1). When sterols are added, the mevalonate is diverted into non-sterol substances owing to sterol-mediated suppression of squalene synthetase and other late enzymes in the sterol synthetic pathway (1).

Translational control of HMG-CoA reductase has been reported previously in avian myeloblasts (28) and in MeV-I cells, a line of CHO cells deficient in HMG-CoA synthase activity (11). In the MeV-I cells, 25-hydroxycholesterol alone reduced the amount of reductase mRNA by 4-fold, but it did not reduce translational efficiency, which only fell in the presence of 25-hydroxycholesterol plus mevalonate. Our current results in non-mutant CHO cells are consistent with these findings.

The observation of mevalonate-mediated translational control is of interest for two reasons: 1) some HMG-CoA reductase mRNAs have complex 5'-untranslated regions (29), which may render them susceptible to regulation at the translational level; and 2) one of the non-sterol products of mevalonate metabolism is isopentenyladenine, which is found adjacent to the anticodon sequence in certain species of tRNA (1, 30). The multiple 5'-untranslated regions in reductase mRNA arise because there are several transcription initiation sites and several splice-donor sites for a large intron in the 5'-untranslated region (29). The utilization of these sites creates two classes of reductase mRNAs. One class, termed class I, contains relatively short 5'-untranslated regions of 68–90 nucleotides in length with no AUG initiator codons upstream of the initiator used for translation of HMG-CoA reductase protein. The other mRNAs, designated class II, contain extremely long 5'-untranslated regions of 354–670 nucleotides in length with as many as 8 AUGs encoding short open reading frames upstream of the reductase initiation site (29). When CHO cells are grown in the absence of sterols under conditions in which the cells make high levels of HMG-CoA reductase, the class I mRNAs predominate, accounting for 70% of the total (29). Because of the complexity of the splicing pattern, it has been difficult to determine which mRNAs predominate when transcription is repressed. It is possible that under these conditions a relatively higher proportion of the mRNAs consists of class II transcripts and that these transcripts are only translated under conditions in which the cells are totally depleted of mevalonate. Such depletion might lead to a fall in isopentenyl-tRNA, thereby triggering translation of the class II mRNAs. Addition of high levels of mevalonate would restore isopentenyladenine and this might suppress translation of the class II mRNAs.

The reason for the complex mevalonate-mediated posttranscriptional control of reductase remains obscure, but a clue emerges from studies of the role of mevalonate in the cell cycle. Studies by Habenicht et al. (31), Quesney-Huneeus et al. (32), and others (33, 34), have shown that cells require a non-sterol substance derived from mevalonate to enter the DNA-synthetic phase (S phase) of the cell cycle. Moreover, HMG-CoA reductase activity rises just before the S phase, even in the presence of sterols (32). It is possible that cells maintain a reserve of poorly translated reductase mRNA during the early phases of the cell cycle and that this mRNA is available to be translated prior to S phase if intracellular mevalonate levels fall. This translation would allow synthesis of the enzyme at a time in the cell cycle when transcription of the HMG-CoA reductase gene might not be adequate. The HMG-CoA reductase molecules would remain active as a result of delayed degradation resulting from the mevalonate deprivation. By this mechanism cells might be able to produce sufficient mevalonate to provide a crucial substance required transiently during the cell cycle. This mechanism would bypass the sterol-dependent control mechanism and thus allow cells to synthesize mevalonate for non-sterol purposes even though intracellular sterols are abundant.

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