Further Characterization of a Chinese Hamster Ovary Cell Mutant Requiring Cholesterol and Unsaturated Fatty Acid for Growth†

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ABSTRACT: In lipid-depleted medium, a Chinese hamster ovary cell mutant that requires cholesterol and unsaturated fatty acid for growth was found to have a much smaller increase in 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity and low-density lipoprotein (LDL) binding activity than normal, wild-type cell [Chin, J., & Chang, T.-Y. (1981) *J. Biol. Chem. 256*, 6304–6310]. We now characterize this mutant further by using compactin which is known to induce an increase in the synthesis of HMG-CoA reductase by a mechanism distinct from the regulatory mechanism controlled by sterol. We found that the mutant, grown in various media, responded to compactin in a manner identical with that of the normal cell. Turnover studies suggested that, although there was an effect on reductase degradation, the effect of compactin on the increase in reductase activity is due mainly to an increase in specific protein synthesis. These data also suggested that the capabilities for synthesis and degradation of HMG-CoA reductase in the mutant are intact and that the observed defect in the mutant is probably due to a specific defect in the regulatory mechanism controlled by sterol. Further analyses indicated that this regulatory mechanism may also control the expression of other early cholesterogenic enzymes, including HMG-CoA synthase and cytosolic acetoacetyl-CoA thiolase, since the increases of these activities in lipid-depleted medium were found to be much less in the mutant than in the normal cell. The increases in each of these activities have been found to revert back to near normal in a spontaneous revertant of the mutant, supporting the idea that all these enzymes are coordinately regulated. Results from assays of stearoyl-CoA desaturase from normal, revertant, and mutant cells implied that the requirement for unsaturated fatty acid in the mutant may be a secondary consequence of the primary defect in the regulation of cholesterol biosynthesis.

The biochemical characterization of mammalian cell mutants with defective lipid metabolism has been useful in delineating and understanding regulatory mechanisms of lipid metabolism (Brown & Goldstein, 1976; Goldstein & Brown, 1977). We have previously isolated and partially characterized a Chinese hamster ovary (CHO)[1] cell mutant that requires cholesterol and unsaturated fatty acids for growth (Chin & Chang, 1981; Limanek et al., 1978). In FCS–M, this mutant grows nearly as well as the normal, wild-type cell. In DeL–M, however, the mutant cell, unlike the normal cell, dies after 2 days, unless the DeL–M is supplemented with both cholesterol and unsaturated fatty acids. We also found that removal of serum lipids from the medium caused a large increase in the activity of HMG-CoA reductase and of LDL binding in the normal cell. Both these increases in activity were much less in the mutant cell. Comparison of the reductase activities from the normal and mutant cells indicated that they were very similar with respect to catalytic and structural properties. These results suggested that the mutation causing the defect was not in the enzyme itself, but possibly in the regulation of HMG-CoA reductase. It was further postulated that HMG-CoA reductase and LDL binding activities were coordinately expressed; the mechanism for controlling the expression of these two activities was defective in the mutant. This idea was supported by the isolation and characterization of a spontaneous revertant of the mutant in which the expression of both HMG-CoA reductase activity and LDL binding activity was found to revert back to near normal (Chin & Chang, 1981).

Compactin is a potent, reversible, and specific inhibitor of HMG-CoA reductase (Endo et al., 1976; Kaneko et al., 1978). Addition of compactin to growth medium causes a large, latent increase in reductase activity, as first demonstrated in human fibroblast cells (Brown et al., 1978). This increase is prevented by cycloheximide, an inhibitor of protein synthesis, implying a process of specific enzyme induction, and it occurs in tissue culture cells incubated in FCS–M or in lipoprotein-deficient serum medium supplemented with LDL (Brown et al., 1978; Goldstein et al., 1979). Thus, compactin can bypass the suppressive regulatory signal exerted by these sterol sources, which normally prevent the increase in the synthesis of HMG-CoA reductase. In this report, we study the effects of compactin on HMG-CoA reductase activity in normal and mutant CHO cells to determine whether the mutant cell reductase would respond to compactin in the same manner as the normal cell reductase. In addition, we further characterize the mutant cell with respect to its ability to increase the activity of two other important cholesterogenic enzymes, HMG-CoA synthase and cytosolic acetoacetyl-CoA thiolase, upon serum lipid depletion (Chang & Limanek, 1980; Miller et al., 1980). We also report our findings on the activity of stearoyl-CoA desaturase, the terminal and rate-limiting component of the fatty acid desaturation system (Strittmatter et al., 1974; Prasal & Joshi, 1979), in the normal, revertant, and mutant cells.

Experimental Procedures

Materials. Biochemicals and most lipids were from Sigma Chemical Co. 25-Hydroxycholesterol (25-OH) from Steraloids and with a purity of approximately 98% was added to tissue culture medium from a stock 0.2% (w/v) solution in dimethyl sulfoxide as previously described (Chin & Chang, 1981). Oleic acid–bovine serum albumin complex was prepared according to a published procedure (Van Harken et al., 1969). The sodium salt of compactin (ML-236B) was pre-

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[1] Abbreviations: CHO cells, Chinese hamster ovary cells; FCS–M, 10% fetal calf serum + F-12 medium; DeL–M, 10% delipidated serum + F-12 medium; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; 25-OH, 25-hydroxycholesterol; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate.

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pared, stored at −20 °C, and added to growth medium from a 0.62 mM stock solution, as described by Brown et al. (1978). Radioactive chemicals were purchased from New England Nuclear.

Cells. As previously described (Chin & Chang, 1981; Limanek et al., 1978), CHO cell cultures were grown as monolayers in 25-cm^2 Falcon tissue culture flasks in F-12 medium (linoleic acid deleted) supplemented with either 10% fetal calf serum (FCS–M) or 10% delipidated serum (DeL–M). Fetal calf serum was delipidated once according to a published procedure (Cham & Knowles, 1976), modified slightly as described previously (Chin & Chang, 1981).

Enzyme Assays. Whole cell homogenates prepared by modifications of a previously described method (Limanek et al., 1978) were used to assay for HMG-CoA reductase activity and for stearoyl-CoA desaturase activity. The cells were collected by scraping and centrifugation and broken by vortexing briefly 3 times during hypotonic shock for 5 min at room temperature in 5 mM potassium phosphate, pH 7.4, and 0.5 mM Na_2EDTA (hypotonic buffer). For HMG-CoA reductase activity, the cell homogenates (0.5–2.0 mg of protein/mL of hypotonic buffer containing 20 mM imidazole, pH 7.4, and 5 mM dithiothreitol) were preincubated at 37 °C for 20 min and then assayed for 60 min at 37 °C as previously described (Chang et al., 1981). Aliquots of 30 and 60 μL of the preincubated homogenates were assayed, and 75 μL of the final reaction mixture was analyzed by thin-layer chromatography. For stearoyl-CoA desaturase, the cell homogenates were brought up to isotonicity with 1 M potassium phosphate buffer, pH 7.4, and aliquots of 35 and 70 μL were assayed for 8 min at 37 °C according to published procedures (Chang & Vagelos, 1976; Oshino et al., 1966). Subcellular fractionation studies have shown that, under the described condition for cell homogenization, over 75% of the microsomal stearoyl-CoA desaturase activity remains in the 900*g* and 10000*g* pellets, suggesting nonspecific association of microsome with the nuclear or mitochondrial fraction during cell breakage. Validity of using the whole cell homogenate as the enzyme source is supported by the finding that the differences and changes in stearoyl-CoA desaturase activity among the normal, revertant, and mutant cells as assayed on the cell homogenates were comparable to the results obtained with the 100000*g* pellet. Protein for the HMG-CoA reductase assay and for the stearoyl-CoA desaturase assay was determined by a microbiuret method (Munkres & Richards, 1965) with bovine serum albumin as the standard. To assay for HMG-CoA synthase and acetoacetyl-CoA thiolase activities, we prepared a cytosolic fraction by the digitonin extraction procedure developed by Mackall et al. (1979). The 25-cm^2 flasks of cells were exposed to 0.3 mL of digitonin buffer at room temperature for 1 min as previously described (Chang & Limanek, 1980). The soluble fractions were collected and centrifuged at 3000*g* for 15 min at 4 °C. An aliquot was frozen at −70 °C and used for acetoacetyl-CoA thiolase assay within a week of storage (Chang & Limanek, 1980), while the rest of the cytosolic fraction was dialyzed for 5 h at 4 °C against a 1000-fold excess of buffer containing 5 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, and 0.5 mM dithiothreitol and then used to determine HMG-CoA synthase activity (Clinkenbeard et al., 1975; Balasubramaniam et al., 1977). Proteins for the synthase or thiolase assays were determined by the Lowry procedure (Lowry et al., 1951).

Control experiments showed that, whenever duplicate flasks of cultures were used in all the assays described in this report, the variation between the duplicate flasks was within 5% from the mean. Therefore, we have employed single flasks of cells throughout this work. Values shown in each figure or table represent the average of results from duplicate assays using cell homogenate prepared from single flasks. Variation of results between duplicates was within 7% from the mean.

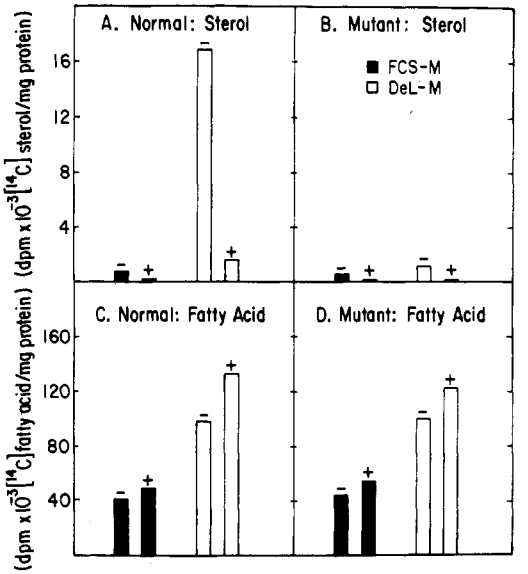

FIGURE 1: Effect of compactin on [^{14}C]acetate incorporation into [^{14}C]cholesterol and ^{14}C-labeled fatty acid in normal and mutant cells in FCS–M or in DeL–M. Normal and mutant cells were plated at 0.20×10^6 and 0.30×10^6 cells per 25-cm^2 flask per 3 mL of FCS–M, respectively. After 2 days of growth with one change of medium, the cells were washed with 6 mL of PBS and then incubated in 5 mL of FCS–M or DeL–M per flask. After 24 h, the medium was replaced with 2 mL of fresh prewarmed medium with or without compactin. After 15–20 min, 8 μL of sodium [1-^{14}C]acetate in H_2O was added for a final concentration of 12 μCi/mL, and the cells were incubated for 1 h at 37 °C. They were then washed 5 times with 5 mL of PBS, dissolved in 1 mL of 2 M NaOH, and analyzed for labeled sterol and fatty acid by thin-layer chromatography (Limanek et al., 1978). The various media were as follows; (■$^-$) FCS–M without compactin; (■$^+$) FCS–M with 2 μM compactin; (□$^-$) DeL–M without compactin; and (□$^+$) DeL–M with 2 μM compactin.

Results

Inhibition of Cholesterol Synthesis by Compactin. Compactin has been shown to inhibit specifically the synthesis of cholesterol from acetate in rat liver homogenates and in various tissue culture cells (Endo et al., 1976; Kaneko et al., 1978; Brown et al., 1978; Goldstein et al., 1979). This was confirmed by the results in Figure 1. In normal cells incubated in Del–M for 24 h, there was a 20-fold increase in the incorporation of labeled acetate into cholesterol (Figure 1A), whereas there was only a 2–3-fold increase in the mutant, confirming our earlier findings (Limanek et al., 1978). The addition of compactin resulted in 92–97% inhibition of cholesterol synthesis but no inhibition of fatty acid synthesis. In fact, in both cell types, there was a small increase in acetate incorporation into fatty acid, similar to that reported by Brown et al. (1978). The significance of this effect is at present unknown. We also found that gas–liquid chromatographic analyses showed that the cellular cholesterol content of normal cells declined from 21 to 9 μg/mg of protein when cells are switched from FCS–M to DeL–M for 24 h. A similar decline was found in the mutant cells (19 μg/mg of protein in FCS–M; 7.8 μg/mg of protein in DeL–M), thus indicating that the much smaller increase in sterol synthesis found in the mutant cells grown in DeL–M was not due to a lack of sterol depletion.

The results of labeled acetate incorporation into fatty acids in the mutant and normal cells grown in FCS–M or in DeL–M

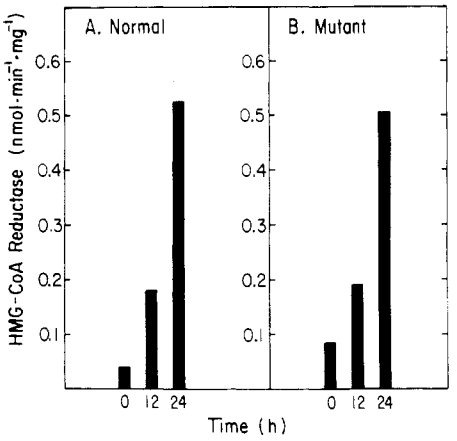

FIGURE 2: Effect of compactin on HMG-CoA reductase activity from normal and mutant cells in FCS–M. Normal and mutant cells were plated at 0.15×10^6 and 0.20×10^6 cells per 25-cm^2 flask per 3 mL of FCS–M, respectively. After 2 days of growth with one change of medium, the cells were washed with 6 mL of PBS, and then 3 mL of fresh prewarmed FCS–M with 2 μM compactin was added. At time 0, 12, and 24 h, cell homogenates were prepared, and HMG-CoA reductase activity was assayed as described under Experimental Procedures.

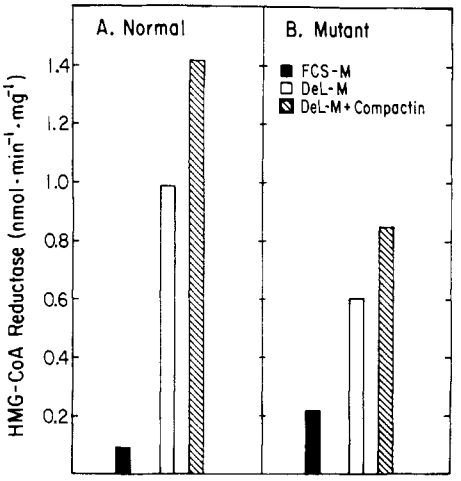

FIGURE 3: Effect of compactin on HMG-CoA reductase activity from normal and mutant cells in DeL–M. Cells were plated and prepared as in Figure 1, except that they were incubated for 24 h in 3 mL of DeL–M with or without compactin or in FCS–M, and the media were replaced with fresh media after 12 h. The cell homogenates were prepared and assayed as in Figure 2. The media used were the following: (■) FCS–M; (□) DeL–M; (▨) DeL–M + 2 μM compactin.

were identical, suggesting that the synthesis of fatty acids in the mutant is not impaired. This conclusion has been confirmed by results which showed that cell extracts from normal and mutant cells have the same glucose-6-phosphate dehydrogenase (Rosenstraus & Chasin, 1975), ATP–citrate lyase (Takeda et al., 1969), and fatty acid synthase (Alberts et al., 1974) activities (data not shown). With the assumption that the syntheses of fatty acid and sterol in intact cells share a common cytosolic pool of acetyl-CoA (Howard et al., 1974), these results also imply that the defects in the mutant are not due to a defect in cytosolic acetyl-CoA production.

Effect of Compactin on HMG-CoA Reductase Activity of Normal and Mutant Cells. Figure 2 presents results of experiments with cells incubated in FCS–M with or without compactin (2 μM) for up to 24 h. After 12 h, there was a 4–5-fold increase in reductase activity over that of the normal cell activity at zero time in both cell types; after 24 h, there was a 10–12-fold increase, resulting in a specific activity of 0.51–0.56 in both cell types. These results confirm an earlier report (Brown et al., 1978) that compactin causes a latent increase in HMG-CoA reductase activity in tissue culture cells. The fact that latent reductase activity is produced in cells by compactin despite the presence of sterol in FCS–M shows that compactin can bypass the suppressive effect of sterol on reductase activity. Furthermore, these results indicate that the mutant is indeed capable of increasing its HMG-CoA reductase activity in the presence of compactin. The result shown in Figure 2 is typical of three separate experiments.

Figure 3 presents results from a similar experiment except that the cells were incubated in DeL–M with or without compactin for 24 h. In normal cells in DeL–M, HMG-CoA reductase activity is already increasing more than 10-fold over the activity of cells grown in FCS–M, so the compactin effect was not as dramatic as that in FCS–M. A similar observation was made in an earlier report on human fibroblast cells (Brown et al., 1978). In mutant cells in DeL–M, the increase in HMG-CoA reductase activity was less than that in the normal cells, confirming our earlier finding (Chin & Chang, 1981). The presence of compactin in DeL–M did, however, elicit an approximately 40% increase in the mutant cell reductase activity, which was very similar to that in the normal cell. In other experiments with both cell types incubated in human or bovine lipoprotein deficient serum medium or lipid-depleted serum medium supplemented with exogenous cholesterol (5 μg/mL) and sodium oleate (5 μg/mL), essentially the same results were obtained. Addition of low concentrations of cholesterol and oleate was shown to support near-normal growth of the mutant in DeL–M (Chin & Chang, 1981; Limanek et al., 1978). This observation, therefore, rules out the possibility that the difference shown in Figure 3 between the normal and mutant cells was due to growth arrest of the mutant in DeL–M.

The results in Figures 1 and 2 suggested that the effect of compactin seems maximal when a suppressor sterol is present in the medium. The effect of compactin on cells grown in DeL–M in the presence of 25-hydroxycholesterol was then determined. This cholesterol analogue is a potent suppressor of HMG-CoA reductase in various types of tissue culture cells, and its mechanism has been reported to involve a decrease in reductase synthesis and/or an increase in reductase degradation (Bell et al., 1976; Chang et al., 1981; Sinensky et al., 1981). The result presented in Figure 4, which is typical of three separate experiments, showed that even in the presence of 25-hydroxycholesterol in DeL–M for 24 h compactin still caused at least a 10-fold increase in HMG-CoA reductase activity in both the normal and mutant cells. This increase was shown to be sensitive to the presence of cycloheximide which confirmed earlier work by Brown et al. (1978) that this increase in reductase activity is dependent on protein synthesis. In addition, from work on rat hepatocytes with antibody prepared against homogeneous HMG-CoA reductase, Edwards et al. (1980) found that, although compactin does cause some activation of reductase activity, most of the increase in activity in the presence of compactin was due to an increase in the number of reductase molecules. Our results, therefore, indicate that the mutant cell has the functional ability to synthesize more reductase protein in DeL–M.

Turnover of HMG-CoA Reductase in the Normal and Mutant Cells. For determination of whether an increase in protein synthesis or a decrease in protein degradation, or both, was the important mechanism for the compactin-mediated increase in reductase activity, turnover studies with cycloheximide[2] were done, and the results are summarized in Table

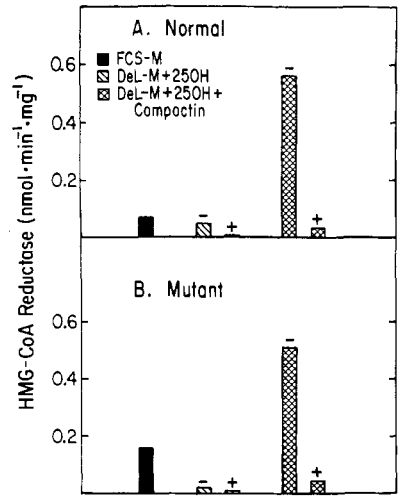

FIGURE 4: Effect of compactin on HMG-CoA reductase activity from normal and mutant cells in DeL–M + 25-hydroxycholesterol in the presence or absence of cycloheximide. Cells were plated and prepared as in Figure 1, except that 3 mL of DeL–M + 25-OH with or without compactin and in the presence or absence of cycloheximide, or 3 mL of FCS–M, was added. Cell homogenates were prepared and assayed for HMG-CoA reductase activity after 24 h as in Figure 2. The various media were following: (■) FCS–M; (▨⁻) DeL–M + 0.5 μg/mL 25-OH without cycloheximide; (▨⁺) DeL–M + 0.5 μg/mL 25-OH with 0.89 mM cycloheximide; (⊠⁻) DeL–M + 0.5 μg/mL 25-OH + 2 μM compactin without cycloheximide; (⊠⁺) DeL–M + 0.5 μg/mL 25-OH + 2 μM compactin + 0.89 mM cycloheximide.

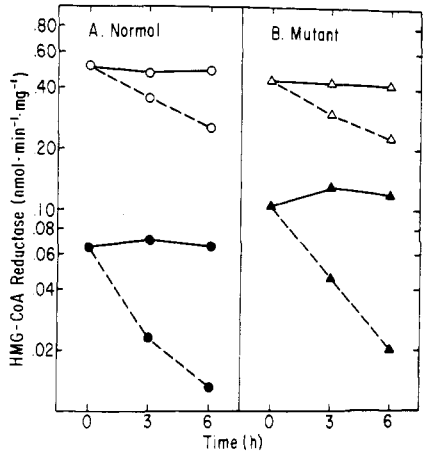

FIGURE 5: Turnover of HMG-CoA reductase in normal and mutant cells grown in FCS–M with or without compactin. Normal and mutant cells were plated and prepared as in Figure 1, except that 3 mL of FCS–M with (open symbols) or without (closed symbols) 2 μM compactin was added, and the cells were incubated at 37 °C for 21 h. The medium in each flask was replaced with 3 mL of fresh, warm, and 5% CO_2-equilibrated medium, and the cells were incubated for 3 h more. Then, at time 0, 50 μL of a cycloheximide solution in PBS was added for a final concentration of 0.89 mM per flask to noncontrol flasks (---). Control flasks received 50 μL of PBS (—). At times 0, 3, and 6 h after the addition of cycloheximide or PBS, cell homogenates were prepared and assayed for HMG-CoA reductase activity as described in Figure 2. (○ or ●) Normal cells in FCS–M with or without compactin, in the presence (---) or absence (—) of cycloheximide; (△ or ▲) mutant cells in FCS–M with or without compactin, in the presence (---) or absence (—) of cycloheximide.

I. A representative figure given in Figure 5 shows typical results obtained from cells grown in FCS–M. In the presence of compactin in sterol-containing medium, FCS–M or DeL–M plus 25-OH, both the normal and mutant cells exhibited a

[2] Control experiments showed that cell cultures treated with cycloheximide for 6 h maintained 90–95% of the total cellular protein content per flask found in zero-time cultures (Chang et al., 1981).

Table I: Rates of HMG-CoA Reductase Activity Turnover in Normal and Mutant Cells in the Presence or Absence of Compactin[a]

	app half-life (h) of HMG-CoA reductase in	
incubation medium	normal cell	mutant cell
FCS–M	2.6, 1.5	2.5
FCS–M + compactin	5.8	6.3
DeL–M + 25-OH[b]	1.94 ± 0.52 (3)	2.4, 2.0
DeL–M + 25-OH + compactin	3.9	4.3
DeL–M[c]	5.81 ± 0.93 (5)	6.2, 5.3
DeL–M + compactin	11.02 ± 0.87 (4)	11.9, 10.2

[a] Apparent half-lives were determined by analyzing the decay in HMG-CoA reductase specific activity with time after addition of cycloheximide in a semilog plot. The data presented in Figure 5 and those from experiments not presented were analyzed by the method of least squares and used to calculate the equation and slope of the line with the best possible fit. From the value of the slope for each experiment, the half-life ($t_{1/2}$) is determined and presented; $t_{1/2}$ values determined from one or two experiments were reported without taking the average; $t_{1/2}$ values determined from three or more experiments were reported as average values followed by the standard error and by the number of experiments performed in parentheses. [b] Cells were plated and prepared as in Figure 5, except that cells were incubated in 3 mL of DeL–M for 21 h, with a fresh medium change after the twelfth hour. Then, 25-hydroxycholesterol was added to the DeL–M for a final concentration of 0.5 μg/mL. After 3 h, cycloheximide or compactin, or both, was added to the flasks, as described in Figure 5. At times 0, 3, and 6 h after the addition of cycloheximide or PBS, cell homogenates were prepared and assayed for HMG-CoA reductase activity as described in Figure 2. Because of the cytotoxic effects of long-term incubation of cells with both 25-hydroxycholesterol and compactin, the effects of compactin and the cholesterol analogue were determined after short-term incubation. [c] Cells were plated and prepared as in Figure 5, except that 3 mL of DeL–M with or without 2 μM compactin was added, and the cells were incubated at 37 °C for 21 h. The medium in each flask was replaced with 3 mL of fresh, warm, and 5% CO_2-equilibrated medium, and the cells were incubated for 3 h more. At time 0, cycloheximide was added, and the cells were treated as in Figure 5.

2–3-fold increase in the apparent half-life of HMG-CoA reductase. These results imply that, although compactin does have an effect on decreasing the rate of degradation of reductase (Alberts et al., 1981), its effect on increasing the rate of synthesis of reductase is much more significant in accounting for the 10-fold increases in both normal and mutant cells seen in Figures 2 and 4. In sterol-free medium such as DeL–M, similar increases (2–3-fold) in the half-life of reductase[3] in both cell types were seen; this slower rate of degradation could account for the 40% increase in reductase activity caused by compactin in Figure 3. Comparison of the apparent half-life of HMG-CoA reductase from normal and mutant cells shows that the rates of degradation in the presence or absence of compactin in FCS–M or DeL–M were the same and were, therefore, not responsible for the difference in reductase activity between the two cell types in the absence of compactin. Thus, the capabilities for protein synthesis as well as protein degradation of HMG-CoA reductase in the mutant cell seem normal.

Defects in the Mutant Cell HMG-CoA Synthase and Cytosolic Acetoacetyl-CoA Thiolase Activities. Evidence previously presented (Chin & Chang, 1981) showed that the

[3] It should be pointed out that the apparent half-life values of HMG-CoA reductase from cells grown in DeL–M reported here (Figure 7 and Table I) are shorter than those previously reported (Chang et al., 1981); this is probably due to the present procedure of refreshing and reequilibrating the cells for 3 h before the addition of cycloheximide and using cells of approximately 4-fold higher density.

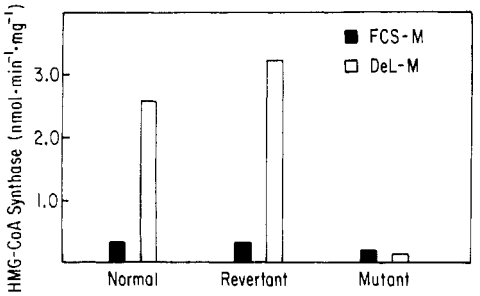

FIGURE 6: HMG-CoA synthase activity of normal, revertant, and mutant cells grown in FCS–M or DeL–M. Normal, revertant, and mutant cells were plated at 0.20×10^6, 0.22×10^6, and 0.30×10^6 cells per 25-cm^2 flask per 3 mL of FCS–M, respectively. After 2 days of growth and a change of medium, the cells were washed with 6 mL of PBS, and then 3 mL of fresh FCS–M or DeL–M per flask was added. After 24 h, the cells were washed and exposed to 0.3 mL of digitonin buffer at room temperature; the soluble fraction was then dialyzed and used to assay for HMG-CoA synthase as described under Experimental Procedures.

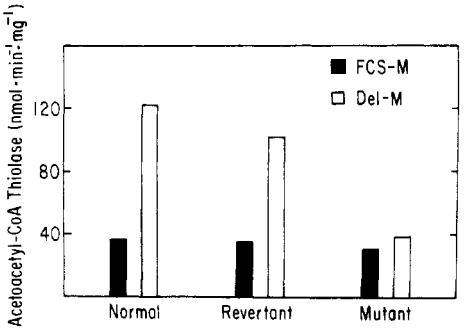

FIGURE 7: Acetoacetyl-CoA thiolase activity of normal, revertant, and mutant cells grown in FCS–M or DeL–M. Cells were plated, prepared, and harvested as in Figure 6, except that the soluble fraction was not dialyzed but immediately stored at −70 °C until assayed. The samples were thawed and assayed within 1 week for cytosolic thiolase activity as described under Experimental Procedures.

increase in the expression of HMG-CoA reductase and LDL binding activities was defective in the mutant. The mutant was further characterized to determine whether the expression of other important cholesterogenic enzymes was also defective. HMG-CoA synthase activities from extracts of normal, revertant, and mutant cells incubated in DeL–M or FCS–M for 24 h were assayed. In the normal and revertant cells grown in DeL–M, there was a 5–10-fold increase in HMG-CoA synthase activity, whereas there was little or no increase in synthase activity in the mutant cell, as shown by a typical result in Figure 6. In experiments not shown, the presence of compactin in FCS–M or in DeL–M had no significant effect on synthase activity in these cell types, indicating that the effect of compactin is specific for HMG-CoA reductase. Parallel studies were done on the expression of cytosolic acetoacetyl-CoA thiolase activity, and the results are presented in Figure 7. Whereas there was a 2–3-fold increase in the thiolase activity of normal and revertant cells incubated in DeL–M, there was no significant increase in the mutant cells. Also, results very similar to those shown in Figures 6 and 7 were obtained when the cells were grown in DeL–M supplemented with cholesterol (5 μg/mL) and oleate (5 μg/mL) and, thus, rule out the possibility that the difference shown in Figures 6 and 7 between the normal and mutant cells was due to growth arrest of the mutant in DeL–M. In experiments not presented, it was found that the increases in activity of both HMG-CoA synthase and acetoacetyl-CoA thiolase in normal and revertant cells incubated in lipid-depleted media were inhibited by cycloheximide, indicating that protein synthesis

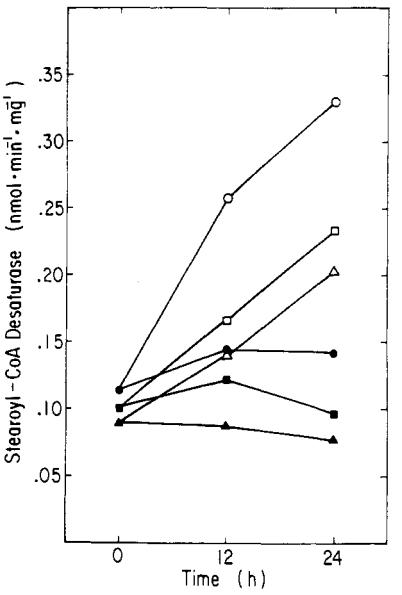

FIGURE 8: Time course of the increase in stearoyl-CoA desaturase activity of normal, revertant, and mutant cells grown in DeL–M over those grown in FCS–M. Cells were plated and prepared as in Figure 6, except that 3 mL of FCS–M was added per 25-cm^2 flask. At time 0, 12, and 24 h, cell homogenates were prepared by hypotonic shock, brought up to isotonicity, and assayed for stearoyl-CoA desaturase activity as described under Experimental Procedures; the fatty acids were extracted, methylated, and analyzed by $AgNO_3$-impregnated thin-layer chromatography. The various cell types and media were the following: (● and ○) normal cells in FCS–M and DeL–M; (■ and □) revertant cells in FCS–M and DeL–M; (▲ and △) mutant cells in FCS–M and DeL–M, respectively.

was necessary for both these increases in activity.

Stearoyl-CoA Desaturase Activity. As reported earlier (Limanek et al., 1978) and based on [^{14}C]acetate incorporation experiments with intact cells as in Figure 1, the defect causing the requirement for unsaturated fatty acids in the mutant was not due to a defect in the synthesis of fatty acids but in the degree of desaturation of total ^{14}C-labeled fatty acids. We now report the time course of the change in stearoyl-CoA desaturase activity in normal, revertant, and mutant cells incubated in FCS–M or DeL–M, as shown in Figure 8. This result was typical of four separate experiments. The specific activity of the normal cell desaturase was shown to be 1.5–2-fold higher than that of the mutant cell desaturase in either FCS–M or DL–M. The specific activity of the revertant cell desaturase was between those of the normal and mutant cells. The increase in desaturase activity between the three different cell types in DeL–M and these cells in FCS–M was, however, about the same (2–3-fold). In addition, from data not presented, we have found that the normal and mutant cells have identical NADH–cytochrome b_5 reductase activity as determined by measuring NADH–ferricyanide reductase activity (Mihara & Sato, 1978) and cytochrome b_5 content (Omura & Sato, 1964). The rate of loss of desaturase activity due to heat inactivation at 43 °C was the same. Although a primary defect in the fatty acid desaturation system cannot be ruled out completely, these results do tend to indicate that the requirement for unsaturated fatty acids in the mutant may be secondary to the primary defect in its ability to express an increase in the activities of the early cholesterogenic enzymes and of LDL binding. A similar tentative conclusion was reached in a study with 25-hydroxycholesterol-treated mouse LM cells (Wattenberg et al., 1979). In support of this hypothesis, we found that the activity increase of stearoyl-CoA desaturase in normal cells grown in DeL–M for 24 h was

prevented by approximately 50% if 20 μg/mL cholesterol was present in DeL–M. The mechanism(s) for the interaction between sterol synthesis and unsaturated fatty acid synthesis is (are) apparently very complex and requires (require) further biochemical investigation.

Discussion

In this report, we have employed a specific agent, compactin, to cause a large latent increase in HMG-CoA reductase in CHO cells. The mechanism of compactin-induced synthesis of reductase is not clear at present; it is possible that the cells are responding to deprivation of another mevalonate-derived substance such as ubiquinone, dolichol, or isopentenyl-tRNA (Brown & Goldstein, 1980). In any event, it is known to be distinct from the regulatory mechanism exerted by lipoprotein-bound cholesterol (Brown et al., 1978). The fact that the mutant cell grown in FCS–M or in DeL–M plus 25-hydroxycholesterol responded to compactin treatment in a manner identical with that of the normal CHO cell (Figures 2, 4, and 5) provides strong evidence that the mutant cell is indeed capable of expressing an increase in HMG-CoA reductase activity. Data from Table I and Figure 5 suggested that the effect of compactin on the increase in reductase activity in both cell types was mainly due to an increase in specific protein synthesis (Brown et al., 1978; Edwards et al., 1980) since its effect on a decrease in reductase degradation (Alberts et al., 1981) was not large enough to account for the observed overall increase in activity. These data and those in Figure 3 also showed that the capabilities for protein synthesis and protein degradation of HMG-CoA reductase in the mutant are probably intact. This implies that the observed defect in reductase activity increase upon serum lipid depletion is probably due to a specific defect in the regulatory mechanism controlled by sterol (Chin & Chang, 1981). Further analyses show that this regulatory mechanism may also control the expression of other early cholesterogenic enzymes including HMG-CoA synthase and cytosolic acetoacetyl-CoA thiolase (Chang & Limanek, 1980), as well as cell-surface LDL-binding activity, since the increases of these activities have been found to be much less in the mutant cell than in the normal cell (Figures 6 and 7). This tentative conclusion is strengthened by the finding that the increases of each of these activities have been found to revert back to near normal in a spontaneous revertant of the mutant. Although the possibility that a structural gene defect of one of the affected enzymes may exist in the mutant, we consider this possibility highly unlikely, because each of these enzymes is known to be a distinct molecular entity (Clinkenbeard et al., 1973, 1975; Edwards et al., 1980; Schneider et al., 1980). No evidence exists which suggests that any two of these enzymes share a common subunit.

Previously, biochemical analyses of two CHO cell mutants resistant to 25-hydroxycholesterol (Chang & Limanek, 1980) also suggested that there may be a common controlling mechanism which regulates the activities of early cholesterogenic enzymes. The significance of this work and the current work is that biochemical genetic evidence for coordinate regulation of various cholesterogenic enzymes has been provided. The nature of such mechanism(s) awaits further exploration at the molecular level.

It is interesting to note that the specific activity of HMG-CoA reductase in the mutant cell incubated in DeL–M for 24 h (Figure 3) was approximately 40–60% of that in the normal cell (Chin & Chang, 1981), while the specific activities of HMG-CoA synthase and acetoacetyl-CoA thiolase in the mutant cell were only 5% and 30% of those, respectively, in the normal cell (Figures 6 and 7). Under the same condition, the rate of sterol synthesis in the mutant, based on [^{14}C]acetate pulse measurement, was found to be only 6% of that in the normal cell (Figure 1).

These data suggest that the increases in activity of early cholesterogenic enzymes other than HMG-CoA reductase are necessary in order to achieve a maximal rate of sterol synthesis in DeL–M. This tentative interpretation relies on the validity of using a [^{14}C]acetate pulse to follow the rate of sterol synthesis in intact cells, since this technique is subject to various limitations (Ryan et al., 1981; Andersen & Dietschy, 1979; Dietschy & Brown, 1974). Further work is necessary to understand this observation and the relative importance of the early cholesterogenic enzymes.

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Synthesis of the Diastereoisomers of 1,2-Dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine and Their Stereospecific Hydrolysis by Phospholipases A_2 and C†

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ABSTRACT: A convenient three-step synthesis of the phosphorothioate analogue of phosphatidylethanolamine is described. The reaction pathway involves the conversion of a 1,2-diacyl-*sn*-glycerol to its corresponding thiophosphoric acid dichloride by using $PSCl_3$ in the presence of a tertiary base. Treatment of the dichloride with ethanolamine results in the formation of a cyclic thiophosphoramidate which, upon acidification, undergoes P–N cleavage, giving rise to 1,2-diacyl-*sn*-glycero-3-thiophosphorylethanolamine. ^{31}P NMR reveals that both diastereoisomers are present in equivalent amounts. It is not possible, however, to separate the two isomers by high-pressure liquid chromatography. ^{31}P NMR and high-pressure liquid chromatography are used to show that phospholipases A_2 and C exhibit absolute and opposite stereoselectivity in the hydrolysis of the pair of diastereoisomers.

Phosphorothioate analogues of nucleotides have proved to be invaluable tools for probing the mechanistic basis of enzyme-catalyzed adenyl- and phosphoryl-transfer reactions and also the role that nucleotides perform in complex biochemical processes (Eckstein, 1975, 1979; Yount, 1975). This suggested to us that phosphorothioate analogues of phospholipids may make important contributions toward our understanding of phospholipid metabolism and of the role that phospholipids play in membrane and cellular function. The consequences of replacing one of the nonbridge oxygens in the phosphodiester linkage of a phospholipid by a sulfur atom are 2-fold. First, the phospholipids will exist as pairs of diastereoisomers due to the chiral phosphorus atom, allowing one to probe the stereoselectivity of specific phospholipases for diastereoisomeric pairs of the thiophospholipids. In certain cases, e.g., phospholipases C and D, it may also enable one to determine the stereochemical outcome of the hydrolysis reaction. Second, it may be expected that the sulfur substitution will make the phospholipid analogues more resistant to enzymatic hydrolysis by phospholipases. Since methods are available for incorporating phospholipids into biological membranes by using either phospholipid exchange proteins (Wirtz, 1974; Zilversmit & Hughes, 1976) or liposomes (Papahadjopoulos et al., 1979), the possible increased stability of the thiophospholipids makes them a potential probe for looking at the role of phospholipid turnover to membrane function.

This paper describes a facile three-step synthesis (without purification of intermediates) of the phosphorothioate analogue of phosphatidylethanolamine.[1] Evidence is presented which shows that phospholipases A_2, which selectively removed the fatty acyl group at C_2, and C, which splits off the complete polar head group, show absolute[2] and, moreover, opposite preferences for one of the diastereoisomers of thiophosphatidylethanolamine.

Experimental Procedures

Materials

Phospholipase A_2 (bee venom, 1200 units/mg), phospholipase C (*Bacillus cereus*, 500 units/mg), 1,2-dipalmitoyl-*sn*-glycerol, and phosphatidylethanolamine were purchased from Sigma. $PSCl_3$ (Alfa) and ethanolamine (Fisher) were distilled before use.

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; phosphatidylethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine; thiophosphatidylethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphorylethanolamine; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[2] Under our assay conditions, once the hydrolysis of the susceptible isomer has finished, there is no observable change in the HPLC profile over a period of 24 h. It is possible, however, that the "so-called" resistant diastereoisomer is being hydrolyzed at a rate undetectable by HPLC.

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