The Dual Role of Mevalonate in the Cell Cycle*

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It is well established that either exogenous or endogenous cholesterol is required for both cell growth and proliferation. This laboratory recently discovered that, in baby hamster kidney-21 cells, independent of its role as a cholesterol precursor, mevalonic acid plays an essential role in S phase DNA replication. It was later shown that isopentenyl adenine, a known product of mevalonate in prokaryotes and lower eukaryotes, is 100 to 200 times more effective than mevalonate in restoring DNA replication in cells in which mevalonic acid synthesis is blocked with the β-hydroxy-β-methylglutaryl-CoA reductase inhibitor, compactin.

The present study was designed to determine the relationship in the cell cycle between the known requirement for cholesterol and the newly discovered effect of mevalonic acid and isopentenyl adenine on S phase DNA synthesis. Employing cells arrested by serum depletion, it was shown that the cholesterol requirement is limited to the early and mid-G1 phases, whereas the isopentenyl requirement is limited at the late G1-S interphase of the cell cycle. The evidence supporting these conclusions involves: first, in serum-arrested cells blocked early in G1 by compactin, only the combination of cholesterol added in early G1 and either mevalonate or isopentenyl adenine in late G1 permitted progression through the G1 and S phase DNA synthesis. Neither isopentenyl adenine added early in G1 nor cholesterol in late G1 was capable of restoring DNA synthesis in this system. Second, in accord with the above formulation, inhibition of cholesterol synthesis with the oxidosqualene cyclase inhibitor, dl-4,4,10-trimethyl-trans-decal-3-ol, affected only the early G1 phase of the cell cycle, but had no late G1 effect on DNA replication.

This laboratory has recently reported that mevalonic acid, independent of its well known function as a cholesterol precursor, plays an essential role in DNA replication which characterizes the S phase of the cell cycle (1-3). This conclusion was based on the finding that baby hamster kidney cells synchronized by the double thymidine block procedure undergo a 5- to 10-fold increase in the activity of HMG-CoA reductase, the enzyme catalyzing mevalonate synthesis, at or just prior to the S phase of the cell cycle. Inhibition of this pulse of HMG-CoA reductase activity by the competitive inhibitor, compactin, totally prevents DNA replication, but has no detectable effect on nonreplicative DNA synthesis, and finally, mevalonate, but not cholesterol, completely restores DNA replication in compactin-treated cells (1). Habenicht et al. (4) and Perkins et al. (5) have confirmed this independent requirement for mevalonate in DNA synthesis. We have subsequently demonstrated that isopentenyl adenine, a known product of mevalonate both in prokaryotic and in lower eukaryotic cells, is more than 100 times more effective than mevalonate in restoring DNA synthesis in cells in which HMG-CoA reductase, and hence DNA replication, is inhibited by compactin (2, 3). Most important, a marked effect of both mevalonate and isopentenyl adenine on DNA replication was observed within minutes of their addition to the cell culture, suggesting that mevalonate, perhaps through the synthesis of isopentenyl adenine or a related compound, may play an initiative role in DNA replication.

In addition to this newly discovered function in DNA replication, mevalonate is well known to serve as a precursor for the cholesterol that is required for the growth of all living cells. Invertebrates, which are incapable of de novo cholesterologenesis, require an exogenous source of cholesterol in order to grow and divide (6). Further, studies using either oxygenated cholesterol derivatives or compactin to inhibit mevalonate and hence cholesterol synthesis have demonstrated that after long term treatment, both cell growth (6-10) and DNA synthesis (8, 10, 12) cease; in several cases these effects could be reversed with both cholesterol and its precursor, mevalonate (7,9, 11, 12). Kaneko et al. (13) have also reported that such long term inhibition of HMG-CoA reductase by compactin results in an inhibition of cell growth but they failed to find an effect on DNA synthesis. Finally, it has been repeatedly shown (14-17) that blockage of cholesterol synthesis with 25-hydroxycholesterol for 1-3 days prevents the typical lectin-induced stimulation of DNA synthesis in lymphocytes. The abbreviations used are: HMG, hydroxymethylglutaryl; LDL, low density lipoprotein; BHK, baby hamster kidney; IPA, isopentenyl adenine; TMD, dl-4,4,10-trimethyl-trans-decal-3β-ol.
is no question, therefore, that cholesterol itself and perhaps other isoprenoids, such as coenzyme Q, are required for cells to complete the cell cycle and to synthesize DNA.

In view of our finding that mevalonate, independent of its function as a cholesterol precursor, causes the prompt restoration of DNA replication in the S phase of the cell cycle (1-3), we have begun studies employing baby hamster kidney cells synchronized by the serum deprivation technique to determine where within the cell cycle the cholesterogenic function of mevalonate is required for cell growth and DNA synthesis. The resulting studies demonstrate that the need for cholesterol, either synthesized from mevalonate or derived exogenously, occurs in the cell cycle early in G1, many hours before DNA replication takes place. Mevalonate in early G1 presumably provides structural cholesterol which permits the cell to pass through G1 and reach the S phase of the cell cycle. However, the present findings also confirm our earlier conclusions that, in order to advance from late G1 into S phase, the synthesis of mevalonate is required to provide the isopentenyl adenosine or related isoprenoids which are required for DNA replication.

These findings indicate that cholesterol, while necessary in early G1 for the passage of cells through G1, has no direct effect on S phase DNA replication. By contrast, quite independent of its function in cholesterogenesis, mevalonate acid probably by means of its isopentenyl adenosine or similar isoprene effect, is required in late G1 for cells to carry out S phase DNA replication.

**EXPERIMENTAL PROCEDURES**

*Reagents*: [methyl-3H]Thd (20 Ci/mM, 1 Ci = 3.7 x 10^10 becquerels), [2-14C]acetate (sodium salt) (51-59 Ci/mM), [1,2-3H]cholesterol (50 Ci/mM), [3-3H]HMG-CoA (40-60 Ci/mM), and Aquasol were obtained from New England Nuclear. DL-[5-3H]Mevalonate acid (dibenzyethyl ethylene diamine salt) was obtained from New England Nuclear. N-[isopentenyl]adenine, dl-mevalonic acid lactone, and NADP⁺ were obtained from Sigma. The ion exchange resin AG 1-X8 (200-400 mesh, formate form) was purchased from Bio-Rad. Inorganic reagents were analytical reagent grade supplied by Baker. Glucose-6-phosphate dehydrogenase was obtained from Sigma. HMG-CoA was from P-L Biochemicals. Dulbecco's essential media with Earle's salts was obtained from the University of California, San Francisco Cell Culture Facility. Media were supplemented with penicillin (1000 units/ml) and fetal calf serum 5%.

Complement was obtained from the Sankyo Co., Ltd. Compacit was brought into solution as described (19). dl-4,4,10P-Trimethyl-trans-decyl-3-fitol was brought into solution as described (19). Isopentenyladenine was brought into solution and stored in 0.1 N NaOH. The ion exchange resin AG 1-X8 (200-400 mesh, formate form) was purchased from Bio-Rad. Inorganic reagents were analytical reagent grade supplied by Baker. Glucose-6-phosphate dehydrogenase was obtained from Sigma. HMG-CoA was from P-L Biochemicals. Dulbecco's essential media with Earle's salts was obtained from the University of California, San Francisco Cell Culture Facility. Media were supplemented with penicillin (1000 units/ml) and fetal calf serum 5%.

*Synchronization of BHK Cells by Serum Deprivation-Repletion*: The method employed for cell synchronization throughout this study involves, as noted above, the use of serum deprivation for 48 h to produce cell arrest in G1. When the media of such cells is supplemented with 5% fetal calf serum, the cells re-enter G1 in a relatively synchronized manner with first order kinetics. To determine the extent of synchronization by this procedure, preliminary studies were carried out using both tritiated thymidine incorporation into DNA and the percentage of cells whose nuclei were labeled with [3H]thymidine as determined by autoradiography.

The results presented in Table 1 demonstrate that at 6 h after supplementation of the media with 5% fetal calf serum, both the level of incorporation of thymidine into DNA and the percentage of labeled nuclei remain unchanged from that at zero time. Between 8 and 12 h, there is a rapid increase of number of cells entering S phase by both criteria, with thymidine incorporation at 12 h increasing 15-fold above the zero hour value and the percentage of cells with labeled nuclei increasing at least 14-fold. At 14 h, there is no further increase in cells entering S phase by either criteria.

These data would indicate that the serum deprivation-repletion technique leads to at least a 90% synchronization of the BHK cells. While a high degree of synchrony was consistently observed throughout these studies, some variation in the time of onset and peak of S phase was observed from experiment to experiment. For this reason, a control sample with serum-arrested synchronized cells is included in each of the experiments reported in this study.

**Effect of Mevalonate Depletion in Late G1 on DNA Synthesis in Cells Synchronized by Serum Depletion**: The initial study was designed to determine whether, as we have previously shown in double thymidine synchronized cells, inhibition of HMG-CoA reductase by compactin in late G1, will

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1. D. Cohen, M. H. Wiley, and M. D. Siperstein, unpublished data.
TABLE I
DNA synthesis and percentage of \(^{3}H\)HdThd-labeled nuclei following serum depletion and repletion of BHK cells

| Time after serum stimulation (h) | \[^{3}H\]Thymidine incorporation into DNA (cpm/mg protein) | Labeled nuclei percentage (%) |
|---------------------------------|----------------------------------------------------------|-------------------------------|
| 0                               | 51.5                                                     | 5-6                           |
| 6                               | 47.5                                                     | 5-6                           |
| 8                               | 94.5                                                     | 15-20                         |
| 12                              | 236.5                                                    | 35-45                         |
| 14                              | 637.7                                                    | 85-90                         |

In Fig. 1, the effect of mevalonate, IPA, and LDL-cholesterol on cells treated with compactin in late G1. Cells were growth arrested by incubating them for 48 h in serum-poor media. They were then stimulated to grow by the addition of fresh media supplemented with 5% fetal calf serum. \(^{3}H\)Thymidine incorporation into DNA was measured every 2 h for a period of 18 h in groups of cells treated as follows: ①, control, no treatment; ②, compactin (comp.) (20 \(\mu M\)) added during late G1 (6 h); ③, compactin (20 \(\mu M\)) and mevalonate (MVA) (0.4 \(\mu M\)) added in late G1 (6 h); ④, compactin (20 \(\mu M\)) and IPA (5.0 \(\mu M\)) added in late G1 (6 h); ⑤, LDL-cholesterol (100 \(\mu g\) of cholesterol/ml) added in late G1 (6 h). In all cases, \(^{3}H\)HdThd was added 30 min prior to cell collection. Bars indicate SEM; n = 3. Chol, cholesterol.

inhibit DNA replication in cells synchronized by the serum deprivation method. As indicated by the results in Fig. 1, the control cells arrested by serum deprivation entered S phase approximately 8 h after the addition of fresh media containing 5% fetal calf serum, and DNA synthesis reaches a maximum between 12 and 14 h. The addition of 20 \(\mu M\) compactin (a concentration that resulted in at least a 95% inhibition of mevalonate synthesis) during late G1 caused a marked inhibition of DNA replication throughout the S phase of the cell cycle. The addition of mevalonate (0.4 \(\mu M\)) 2 h before the initiation of the normal S phase completely reversed this compactin-induced inhibition of DNA replication (Fig. 1).

To determine whether the compactin-induced block of DNA replication might be the result of cholesterol depletion rather than the absence of mevalonate itself, the ability of LDL cholesterol (100 \(\mu g\)/ml) to reverse this inhibition of DNA replication was next evaluated. When added during late G1, at the same time as compactin, cholesterol at a final concentration of 100 \(\mu g\)/ml was clearly unable to overcome the inhibition of DNA synthesis produced by compactin. Lower concentra-
cycle. The effect of compactin added in early GI was therefore examined. As indicated in Fig. 3, when mevalonate synthesis was prevented throughout GI, no significant incorporation of thymidine into DNA above the basal level occurred during the expected S phase of the normal cell cycle. The effect of compactin added at zero time, i.e. in early GI, is more pronounced than when added at 6 h, i.e. in late GI. As also indicated in Fig. 3, mevalonic acid, when added during early GI, reversed the compactin-induced inhibition of DNA replication. Once again, LDL-cholesterol added in early GI was totally ineffective in overcoming the inhibition of DNA synthesis. The major finding in this experiment, however, was that isopentenyl adenine, which as noted above, when added late in GI, would largely reverse the compactin inhibition of DNA replication, was completely incapable of restoring DNA synthesis to compactin-treated cells when added in early GI.

**Effect on DNA Replication of Cholesterol Added in Early GI and Mevalonate or IPA Added in Late GI to Compacted-treated Cells**—In order to distinguish the distinct roles of mevalonate in the cell cycle which were implied by the previous experiments, cells were again treated with compactin throughout GI, and LDL-cholesterol and mevalonate were added sequentially, i.e. the cholesterol in early GI and the mevalonate in late GI. As indicated by the results in Fig. 4, this combination of cholesterol and mevalonate caused total rescue of DNA synthesis in compactin-treated cells.

By contrast, if this experiment is repeated, omitting the cholesterol treatment in early GI and adding mevalonate only in late GI, the peak of DNA synthesis is delayed by about 4 h, i.e. from 14 h to approximately 18 h, after removal of the serum block. This finding would suggest that when cells are deprived early in GI of both cholesterol and its precursor, mevalonate, passage from early to late GI is retarded until mevalonate is added to the compactin-treated cells, thereby providing cholesterol that is required for optimum cell growth. When such cells proceed to the delayed late GI, the added mevalonate, presumably by providing isopentenyl adenine or related isoprenes, permits the initiation of DNA replication.

These studies were extended in a separate experiment in which the ability of cholesterol, mevalonate, and, in particular, isopentenyl adenine to reverse the compactin inhibition of S phase DNA synthesis was further determined in cells treated with compactin at zero hour.

As indicated by the data in Table II, the peak of S phase DNA synthesis occurred in the control cells at 12 h. The degree of thymidine incorporation into DNA was not significantly affected in these cells by the addition of mevalonate, LDL-cholesterol, or cholesterol in ethanol. While not included in our study, we repeatedly find that, as expected, IPA added at zero time to control cells has no effect on DNA synthesis. The addition of compactin at zero time led to a marked decrease in S phase DNA synthesis which was most apparent at 12 h (Table II).

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**Table II**

Reversal of compactin inhibition of DNA replication by addition of cholesterol in early GI and mevalonate or IPA in late GI. Cells were arrested by serum deprivation and after 48 h were stimulated to grow by adding media supplemented with 5% fetal calf serum. The serum was incubated 30 min prior to cell collection in [3H]thymidine (2.0 μCi/ml) for determining thymidine incorporation into DNA. Cells were divided into the following 4 groups and collected every 2 h for a period of 18 h: control, no treatment; compactin (comp) (20 μM) added in early GI (0 h); compactin (20 μM), LDL-cholesterol (100 μg of cholesterol/ml) added in early GI; mevalonate (MVA) (6.4 μM) added in late GI (6 h); compactin (20 μM) added in early GI, mevalonate (0.4 μM) added in late GI (6 h). Bars represent SEM; n = 3, Chol, cholesterol.

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Fig. 4. The effect of LDL-cholesterol added in early GI and mevalonate added in late GI on DNA synthesis of compactin-treated cells. Serum-arrested cells were stimulated to grow by the addition of fresh media supplemented with 5% fetal calf serum. Cells were incubated 30 min prior to cell collection in [3H]thymidine (2.0 μCi/ml) for determining thymidine incorporation into DNA. Cells were divided into the following 4 groups and collected every 2 h for a period of 18 h: control, no treatment; compactin (comp) (20 μM) added in early GI (0 h); compactin (20 μM), LDL-cholesterol (100 μg of cholesterol/ml) added in early GI; mevalonate (MVA) (6.4 μM) added in late GI (6 h); compactin (20 μM) added in early GI, mevalonate (0.4 μM) added in late GI (6 h). Bars represent SEM; n = 3, Chol, cholesterol.

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Fig. 3. Compactin added in early GI prevents cells from entering S phase, this effect is reversible by mevalonate but not by IPA. Serum-arrested cells were stimulated to re-enter the cell cycle by the addition of fresh media supplemented with 5% fetal calf serum. Thymidine incorporation into DNA was determined by incubating cells for 30 min in [3H]thymidine (2.0 μCi/ml). Cells were divided into the following groups and collected every 2 h for a period of 18 h: control, no treatment; compactin (comp) (20 μM) added in early GI (0 h); mevalonate (MVA) (0.4 μM) and compactin (20 μM) added in early GI (0 h); IPA (5 μM) and compactin (20 μM) added in early GI (0 h); LDL-cholesterol (100 μg of cholesterol/ml) and compactin (20 μM) added in early GI. Bars represent SEM; n = 3, Chol, cholesterol.
at the 8th and 12th h after release of the serum arrest. Neither cholesterol nor IPA added at zero time had a significant effect upon DNA replication, however, in conformity with the results shown in Fig. 1, mevalonate added at zero time caused a significant stimulation of DNA synthesis, which in this experiment was most apparent at 16 h. The major aim of this experiment was to determine whether cholesterol added either as lipoprotein bound or dissolved in ethanol, plus mevalonate or isopentenyl adenine added at 6 h would reverse the compactin inhibition of S phase DNA synthesis. The results in Table II demonstrate that, as compared to the insignificant effect of added cholesterol alone, the further addition of mevalonate or of IPA at 6 h caused a highly significant increase in DNA synthesis both at 12 and 16 h after removal of the serum arrest.

As shown in Table III, the ability of cholesterol added at early G1, plus IPA added at late G1, to restore DNA replication in compactin-treated cells is confirmed by autoradiographic assessment of the percentage of nuclei labeled by ['H]thymidine.

The results in Table II also show that, as expected, the simultaneous addition of IPA or LDL-cholesterol to the compactin-treated cells did not result in a stimulation of DNA synthesis. Finally, if LDL and mevalonate are both added at 6 h, a stimulation of DNA synthesis occurs; however, the maximum thymidine incorporation into DNA is observed at 16, rather than 12, h. This lag, as noted above, probably represents the delay in initiation of G1; until the time that LDL-cholesterol is added. In conformity with this assumption, the addition of mevalonate alone at 6 h likewise stimulated DNA synthesis to a somewhat lesser extent, with a comparable shift in the maximum DNA synthesis. Perhaps due to redistribution of isoprene precursors (see "Discussion") in this experiment, the addition of LDL-cholesterol at zero time resulted in a delayed, partial restoration of DNA synthesis at 16 h.

Deprivation of Cholesterol during Arrest of Cell Growth—Studies were next carried out to determine the effect of depriving cells of endogenous as well as of exogenous cholesterol during the period of serum depletion arrest. For this study, cells were placed in media containing 0.1% fetal calf serum. Incorporation of ['H]thymidine into DNA was determined at 0 and 14 h after the addition of serum. Number of experiments = 3.

**TABLE III**

| Additions | Time after addition of serum (h) | ['H]Thd incorporation into DNA (mean ±SE) | Labeled nucleoli % |
|-----------|----------------------------------|------------------------------------------|-------------------|
| Control   | 0 h                              | 27.3 ± 1.2                               | 4-5               |
|           | 14 h                             | 341.6 ± 0.9                               | 75-85             |
| Compactin | 14 h                             | 178.8 ± 4.5                               | 20-30             |
|           | 14 h                             | 243.1 ± 0.7                               | 30-35             |
| Compactin + cholesterol-LDL | 14 h | 362.4 ± 20.4 | 60-70 |

**TABLE IV**

| Treatment | ['H]Thd incorporation into DNA at: | cpm x 10^3/μg protein |
|-----------|-----------------------------------|-----------------------|
|           | 0 h                               | 28.9                  |
|           | 8 h                               | 182.6                 |
|           | 12 h                              | 424.6                 |
|           | 16 h                              | 294.7                 |
| Control   | (48 h 0.1% fetal calf serum)       | Control (48 h 0.1% fetal calf serum) |
|           |                                   | Compactin (48 h 0.1% fetal calf serum; last 24 h compactin, 2.5 μM) |
|           |                                   | 32.4                  |
|           |                                   | 37.6                  |
|           |                                   | 187.8                 |
| Compactin | + mevalonate (48 h 0.1% fetal calf serum) | Compactin + mevalonate (48 h 0.1% fetal calf serum) |
|           |                                   | 176.6                 |
|           |                                   | 383.8                 |
|           |                                   | 237.6                 |
| Compactin | + IPA (48 h 0.1% fetal calf serum; last 24 h compactin + LDL-cholesterol) | Compactin + LDL-cholesterol (48 h 0.1% fetal calf serum; last 24 h compactin + LDL-cholesterol) |
|           |                                   | 197.8                 |
|           |                                   | 414.6                 |
|           |                                   | 246.5                 |
nosis, with the maximum effect occurring 4 h after release of the serum block, that is, at the time in the cell cycle when cholesterogenesis normally reaches its maximum (4 h, control 38.5, TMD 6.7 pmol/min of acetate incorporated into cholesterol/mg of protein; 8 h, control 10.8, TMD 4.4, pmol/min of acetate incorporated into cholesterol/mg of protein). Studies, the data for which are not presented, have shown that under the conditions employed, TMD has no effect upon HMG-CoA reductase activity.

The effect on DNA synthesis of inhibiting cholesterogenesis with TMD in either early or late G1 was next determined. As indicated in Table V, when TMD was added at zero time, that is, at the beginning of G1, subsequent DNA replication is greatly inhibited. By contrast, when the TMD inhibition of cholesterogenesis is induced late in G1, DNA replication was totally unaffected. These results further support the conclusion that cholesterol is required presumably for the synthesis of structural membranes in the early and mid-G1 phases of the cell cycle, but that cholesterol itself plays no direct role in S phase DNA replication. This conclusion is strongly supported by the finding (Table V) that the addition of cholesterol-ethanol, independent of its role of providing endogenous cholesterol, either as LDL or in ethanol, during early G1 completely reversed the inhibition of S phase DNA synthesis caused by TMD. By contrast, as shown in Fig. 5, mevalonate added to TMD-treated cells early in G1, did not reverse the TMD-induced inhibition of DNA synthesis.

Finally, as demonstrated in Fig. 6, if synchronized BHK cells are treated with TMD early in G1 to inhibit cholesterogenesis and with compactin in late G1 to inhibit mevalonate synthesis, the resulting inhibition of DNA replication can be overcome by a combination of cholesterol added in early G1, and mevalonate replacement in late G1. Neither cholesterol nor mevalonate alone was capable of reversing the inhibition of DNA replication produced by the double block with TMD and compactin.

**DISCUSSION**

HMG-CoA reductase serves at least two obligatory functions in the cell cycle. First, as shown by many previous studies, HMG-CoA reductase provides the mevalonate that is needed for the synthesis of cholesterol which, in turn, is required for the production of cell membranes and, hence, cell growth. (6-17). Secondly, however, we have recently demonstrated that mevalonate, quite independent of its function as a cholesterol precursor, plays an essential role in the initiation of DNA replication (1, 3). We have further provided evidence that isopentenyl adenine, or a related isoprene purine, may mediate this effect of mevalonate in the cell cycle (2, 3).

The primary purpose of the present study has been to confirm and extend the evidence that HMG-CoA reductase is required early in the cell cycle at mevalonate in the cell cycle and to determine specifically where within this cycle the cholesterogenic function of mevalonate is required. The major finding of the present study is that cholesterol, either endogenously synthesized from mevalonate or supplied exogenously, is specifically required early in G1 to permit the cell to undertake the growth phase that is characteristic of passage from early to late G1 in preparation for DNA replication. By contrast, as we have previously shown (1), cholesterol itself appears to play no direct function in initiation of DNA replication during the S phase of the cell cycle. The present results also confirm our finding that mevalonate, independent of its role of providing endogenous cholesterol for the structural requirements of the cell, serves a

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**TABLE V**

| Condition            | TMD (0 h) | Cholesterol-LDL (0 h) | Cholesterol-ethanol (0 h) | TMD (6 h) | Cholesterol-LDL (6 h) | Cholesterol-ethanol (6 h) |
|----------------------|-----------|-----------------------|---------------------------|-----------|-----------------------|---------------------------|
| cpm × 10^7/g protein | 37.4      | 246.7 ± 0.9           | 163.8 ± 2.8               | 278.9 ± 0.3 | 237.6 ± 4.2          | 251.4 ± 3.2               |
| Control              |           |                       |                           |           |                       |                           |
| TMD (0 h)            | 0         |                       |                           | 302.6 ± 1.6 | 316.5 ± 0.5          |                           |
| TMD (6 h)            | +         |                       |                           |           |                       |                           |
| + Cholesterol-LDL    | 6         |                       |                           |           |                       |                           |
| + Cholesterol-ethanol| 8         |                       |                           |           |                       |                           |
| + Mevalonate (MVA)   | 14        |                       |                           |           |                       |                           |

**Fig. 5.** Effect of TMD added in early G1 on subsequent DNA synthesis. Serum-arrested cells were stimulated to grow by the addition of serum. [3H]dThd incorporation into-DNA was determined 12, 16, and 18 h after the addition of serum in cells treated as follows: □, control, no treatment; □, TMD (20 μg/ml) added in early G1 (0 h); ■, TMD (20 μg/ml) and mevalonate (MVA) (0.4 μM) added in early G1 (0 h); ○, TMD (20 μg/ml) and LDL-cholesterol (100 μg of cholesterol/mg of protein) added in early G1 (0 h). Bars represent SEM; n = 3, chol, cholesterol.

**Fig. 6.** The effect of LDL-cholesterol and mevalonate on cells treated with TMD and compactin on subsequent DNA synthesis. Serum-arrested cells were stimulated to grow by the addition of fresh media supplemented with 5% fetal calf serum. [3H]dThd incorporation into-DNA was determined 14, 16, and 18 h after the addition of serum in cells treated as follows: □, control, no treatment; □, TMD (20 μg/ml) added in early G1 (0 h) and compactin (20 μg/ml) added in late G1 (6 h); ○, TMD (20 μg/ml) and LDL-cholesterol (100 μg/ml) added in early G1 (0 h); □, compactin (20 μg/ml) and mevalonate (MVA) (0.4 μM) added in late G1 (6 h). Bars represent standard error of the mean, n = 3. Chol, cholesterol.
The dissociation of the two functions of mevalonate during the GI-S phase of cell cycle could largely be replaced by isopentenyl adenine. In this regard, Brown and Goldstein have shown that after maximum cholesterol-induced feedback inhibition of HMG-CoA reductase and hence mevalonate production, as would be predicted, the presence of TMD early in G₁ and compactin in late G₁ results in an inhibition of DNA synthesis that can only be reversed by the combined addition of cholesterol in early G₁ and mevalonate in late G₁.

Taken together, these findings strongly support our previous conclusion that the synthesis of mevalonate performs two distinct and essential functions in the cell cycle. First, mevalonate serves a relatively long term role as a precursor of the cholesterol that is necessary for membrane structure, for cell growth, and for the resulting progression of the cell through the G₁ phase of the cell cycle. Consistent with such a formulation, this function of mevalonate can be completely replaced by exogenous cholesterol. It is very likely that with prolonged cell propagation in the presence of compactin, deprivation of other structural and functional isoprenoids, such as dolichol and coenzyme Q, would become apparent. The present findings indicate, however, that cholesterol, but not other isoprenes such as dolichol and coenzyme Q, is limiting for cell growth during the 8-10 h of the single G₁ phase characteristic of the BHK cell examined in these studies.

The experiment in Table IV further demonstrates that cholesterol is needed not only during the G₁ phase of cell growth, but is also required during the period of cell arrest (Ga). In this experiment, when cells were deprived by compactin treatment of endogenous cholesterol during serum depletion arrest, restoration of cholesterogenesis by the addition of 5% fetal calf serum did not result in normal progression through G₁; and S phase DNA synthesis was delayed by at least 8 h. Since the addition of either mevalonate or cholesterol to compactin-treated, growth-arrested cells completely restored normal G₁ progression, it is clear that cholesterol must serve an essential function either for cell growth or membrane replenishment, even during cell arrest or the G₁ phase of the cell cycle.

In addition to providing the cholesterol required for cell growth, this and our previous studies have demonstrated that mevalonate, completely independent of its function as a cholesterol precursor, is required in late G₁ to initiate DNA replication. In contrast to the relatively long term requirement for cholesterol, amounting to several hours in the cell cycle, mevalonate can, as shown in previous studies, initiate DNA replication in late G₁ within minutes after its addition.

Isopentenyl adenine can largely or completely replace the late G₁-S phase functions of mevalonate; and it is likely, therefore, as we have previously suggested (2,3), that isopentenyl adenine or a closely related purine may mediate the effect of mevalonate in initiating DNA replication. In certain of the experiments reported in the present study, isopentenyl adenine, while causing a marked stimulation of DNA replication in compactin-inhibited cells, did not result in full restoration of DNA replication. This finding raises the possibility that after deprivation of mevalonate for several hours, in addition to isopentenyl adenine, other isoprenes may play a role in achieving maximal DNA replication.

In this regard, Brown and Goldstein have shown that after maximum cholesterol-induced feedback inhibition of HMG-CoA reductase, the addition of mevalonate results in a significant further inhibition of this enzyme. On the basis of this finding, they have postulated that a nonsterol derivative of mevalonate acts cumulatively with cholesterol to inhibit the relatively rapid function in initiating S phase DNA synthesis. Moreover, this latter role of mevalonate in DNA replication could largely be replaced by isopentenyl adenine.

Our previous studies of the role of mevalonate in the cell cycle made use of BHK cells synchronized by the double thymidine block procedure. This technique causes a block in cell replication in the G₁-S interphase and, therefore, precludes studies of the entire G₁ phase of the cell cycle. In the present studies, we employed the serum-depletion technique, which arrests cells in early G₁, thereby allowing a detailed examination of the role of mevalonate synthesis both in early and late G₁, as well as in the S phase of the cell cycle. Employing cells synchronized by the serum depletion-repletion method, we could fully confirm our earlier findings that mevalonate is required in late G₁ for cells to initiate S phase DNA replication. Additionally, in this experiment, isopentenyl adenine could once again largely replace mevalonate in initiating S phase DNA replication and proved again to be significantly more potent than mevalonate in stimulating DNA replication in cells in which mevalonate synthesis was blocked with compactin. By contrast, the addition of cholesterol in late G₁ had no effect on DNA replication in the compactin-treated cells. This experiment once more documents the requirement for mevalonate but not cholesterol for the initiation of DNA replication in cells that have reached the late G₁ phase of the cell cycle.

Studies next were focused on the role of mevalonate in early G₁. When mevalonate synthesis was inhibited throughout G₁ by the addition of compactin at the time of the removal of the serum depletion arrest, S phase DNA replication, which usually begins 8-10 h later, was completely prevented. The presence of mevalonate in early G₁ fully reversed the compactin inhibition of S phase DNA replication; however, isopentenyl adenine was totally incapable of replacing this mevalonate requirement in early G₁. This finding provided the first direct evidence that mevalonate plays at least two roles in the cell cycle: one during early G₁ that cannot be replaced by isopentenyl adenine and another in late G₁, which can be largely reproduced by isopentenyl adenine at 1/200 the concentration of mevalonate. Direct evidence that the early G₁ function of mevalonate is to serve as a cholesterol precursor is provided by the finding that the addition of lipoprotein-cholesterol during early G₁ could completely replace the requirement for mevalonate, whereas in late G₁, cholesterol is incapable of assuming the role of mevalonate in DNA replication.

Consistent with this conclusion was the observation that when cells treated with compactin in early G₁ were not rescued with mevalonate until late G₁, the peak of DNA replication was delayed for approximately 4-6 h beyond its normal occurrence. Presumably under these circumstances, passage through G₁ was delayed until amounts of endogenous cholesterol adequate for cell growth were synthesized from the mevalonate. The added mevalonate was thereafter available to serve a second function, namely the initiation of DNA replication, which was maximal at the 18th instead of the normal 14th h after release of the serum block.

The dissociation of the two functions of mevalonate during the G₁ phase of cell replication was further documented by the use of a specific inhibitor of oxidosqualene cyclase, TMD, which prevents the synthesis of cholesterol but not mevalonate (19). Consistent with a cholesterol requirement in early, but not in late, G₁, the addition of TMD in early G₁ prevented subsequent DNA replication, whereas TMD was totally without effect on DNA replication when added in late G₁. Moreover, cholesterol completely reversed the effect of TMD on DNA synthesis if the cholesterol was added in early G₁, but could not reverse the effect of TMD if added in late G₁. In view of the main site of action of TMD, mevalonate alone could not reverse the TMD effect. The dual effect of mevalonate on the cell cycle could be further confirmed by the combined use of TMD to inhibit cholesterol synthesis and compactin to inhibit HMG-CoA reductase and hence mevalonate production. As would be predicted, the presence of TMD early in G₁ and compactin in late G₁ results in an inhibition of DNA synthesis that can only be reversed by the combined addition of cholesterol in early G₁ and mevalonate in late G₁.
HMG-CoA reductase (18). They were not able to demonstrate such a feedback role for such mevalonate products as squalene, ubiquinone, or dolichol. Whether isopentenyl adenine can serve this function in suppressing HMG-CoA reductase remains to be determined.

Brown and Goldstein (11) have further provided evidence that a multivalent feedback regulation of HMG-CoA reductase would allow the cell, in the presence of inhibiting concentrations of cholesterol, to divert a diminished supply of isoprene units selectively to maintain the synthesis of essential mevalonate products such as ubiquinone and dolichol, and, perhaps, IPA. Since, in our studies, only relatively small amounts of isopentenyl adenine are needed to stimulate DNA synthesis to maximal levels, it is unlikely that even under conditions of feedback inhibition of cholesterol, mevalonate would be limiting for IPA synthesis. The role of IPA in such a multivalent feedback system is now under study.

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