The effect of mevalonic acid deprivation on enzymes of DNA replication in cells emerging from quiescence

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We have investigated the biochemical basis of the mevalonate dependence of DNA replication. Stimulating quiescent rat hepatoma cells to proliferate in the presence of compactin, an inhibitor of mevalonate synthesis, prevented DNA replication in as many as 80% of these cells. The percentage of cells that failed to replicate DNA increased with the increased duration of quiescence. Aphidicolin-sensitive DNA polymerase and ornithine decarboxylase activities were selectively decreased in compactin-treated cells, whereas RNA and protein synthesis, the level of dihydrofolate reductase and aphidicolin-resistant DNA polymerase activity were unaffected. Adding putrescine, the product of ornithine decarboxylase and the precursor of other polyamines, did not restore DNA replication. Our results demonstrate that the decreased activities of at least two DNA-replication enzymes are among the proximal causes of the failure of mevalonate-deprived cells to synthesize DNA. More importantly, our data indicate that a mevalonate-dependent factor(s) is progressively depleted during quiescence, and that inability to resynthesize this factor(s) may be the ultimate cause of the failure of resting cells to replicate DNA when stimulated to proliferate in the absence of mevalonate.

INTRODUCTION

That cholesterol is essential for cell growth and DNA replication was first shown by Chen *et al.* [1] and Brown & Goldstein [2]. Subsequent work from our laboratory first revealed that mevalonic acid, independent of its function as a precursor of cholesterol, is required concomitantly for S-phase DNA synthesis [3,4]. Thus, when quiescent BHK cells were stimulated to proliferate in the presence of compactin, an inhibitor of mevalonate biosynthesis [5], incorporation of thymidine during the ensuing S phase was decreased [3]. Exogenous mevalonate, but not exogenous cholesterol, abolished the decrease in DNA synthesis [3,4]. Extension of these findings to other normal and malignant cells [6–10], either actively proliferating or emerging from quiescence, and to yeast [11], confirmed the dependence of DNA replication on mevalonic acid.

The mechanism by which mevalonate deficiency blocks DNA replication is unknown. Recent studies have revealed that a small subset of proteins is covalently modified by mevalonate-derived ligands [12]. These prenylated polypeptides include lamin B and lamin A, the γ subunits of G-proteins, small GTP-binding proteins and *ras* proteins (reviewed in [13]; [14–17]). Notably, guanine-nucleotide binding proteins have been implicated in growth control [18,19]. However, no involvement of any of the identified prenylated proteins in the failure of mevalonate-deprived cells to replicate DNA has been demonstrated.

To define the biochemical basis of the failure of mevalonate-deficient cells to replicate DNA, we studied the effect of compactin on key DNA-replication enzymes in rat hepatoma cells emerging from quiescence. Our results demonstrate that mevalonate insufficiency affects two key enzymes required for DNA replication, and that an immediate cause of the failure of DNA synthesis is the decreased activity of aphidicolin-sensitive DNA polymerases. In addition, our data indicate that competency to replicate DNA in the absence of mevalonate synthesis is progressively lost during quiescence.

MATERIALS AND METHODS

Reagents

[³H]Thymidine (20 Ci/mmol), [³H]TTP (79.8 Ci/mmol), [³H]uridine (44.4 Ci/mmol), and L-[³H]amino acids were obtained from New England Nuclear. DL-[1-¹⁴C]Ornithine hydrochloride (40 Ci/mmol) was from Amersham. Unlabelled deoxyribonucleoside triphosphates, ATP and dideoxythymidine 5'-triphosphate (d₃TTP) were from Pharmacia-P.L. Biochemicals. Thymidine, uridine and DL-mevalonic acid lactone were from Sigma. Aphidicolin was from Calbiochem. Gapped double-stranded calf thymus DNA was a gift from Dr. L. Loeb (Department of Pathology, University of Washington). Compactin and mevinolin [5,20] were generously given by Dr. Muth of Sanyo Co. and Dr. A. Alberts of Merck, Sharp and Dohme Research Laboratories respectively. Each drug was dissolved in 0.1 M-NaOH by heating at 50 °C for 120 min. The pH of the solution was adjusted to 7.4 with HCl and the concentration adjusted to 2 mM. The stock solution was sterilized by filtration and stored at –20 °C. Dialysed fetal-calf serum was prepared by dialysis of 50 ml portions against 5 × 1 litre of phosphate-buffered saline (PBS) at 4 °C for 3 days. The final media contained approx. 1.3–2.0 mg of cholesterol/dl.

Cell culture

HTC cells [21] were obtained from the Cell Culture Facility, University of California, San Francisco. Cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 3 g of glucose/l, 2 mM-glutamine, 20 units of penicillin/streptomycin and 5% fetal-calf serum. Experiments measuring incorporation of [³H]thymidine, [³H]TTP, [³H]uridine, and [³H]amino acid were carried out in 35 mm-diam. Petri dishes inoculated with 1 × 10⁵ cells in 1 ml of medium. For preparation of cell extracts, 150 mm-diam. Petri dishes were inoculated with 5 × 10⁵–1.5 × 10⁶ cells in 20 ml of medium.

Cells were made quiescent by deprivation of isoleucine and

Abbreviations used: d₃TTP, dideoxythymidine 5'-triphosphate; PBS, phosphate-buffered saline; DHFR, dihydrofolate reductase; ODC, ornithine decarboxylase; HMG-CoA, 3-hydroxyl-3-methylglutaryl-CoA.

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glutamine [22]. Approx. 72 h after inoculation, cells were washed twice with PBS and then incubated in isoleucine- and glutamine-free Dulbecco's modified Eagle's medium containing 5% dialysed fetal calf serum for 48 or 72 h. Quiescent cells were stimulated to proliferate by returning them to complete medium. At stimulation, fewer than 5% of cells incorporated [3 H]thymidine during a 30 min pulse, as determined by autoradiography (see below). At the peak of S phase, 57% of cells incorporated label during a 30 min period.

DNA, RNA and protein synthesis

Cells were incubated with 2 μ M-[3 H]thymidine (5 μ Ci/ml), 2 μ M-[3 H]uridine (5 μ Ci/ml) or [3 H]amino acid mixture (0.5 μ Ci/ μ l) for 15 min, washed with 2 $\times$ 1 ml of cold PBS, and held for 30 min at 4 °C in 1 ml of cold 5% trichloroacetic acid. The cells were washed with 2 $\times$ 1 ml of cold 95% ethanol. After drying, cells were solubilized in 1 ml of 2% SDS. A 0.6 ml sample was used to determine incorporation of radiolabelled precursor, and one of 0.2 ml to measure protein concentration by the method of Lowry *et al.* [23], with BSA as a standard. Incorporation of the radioactive precursors was linear for 120 min. Autoradiography was as previously described [4]. Unless otherwise stated, data presented for these and the following experiments are means $\pm$ S.E. of triplicate determinations in a representative experiment.

Isolation of nuclei and nuclear DNA synthesis

Nuclei were prepared as previously described [24]. Briefly, cells were rinsed with 1 ml of wash buffer (30 mM-Hepes, pH 8.0, 200 mM-sucrose, 5 mM-NaCl, 5 mM-MgCl₂ and 1 mM-CaCl₂). Nuclei were prepared *in situ* by treatment for 30 s with 1 ml of wash buffer containing 0.5% Nonidet P-40. After gently rinsing with 2 ml of wash buffer, nuclear DNA synthesis was assayed by adding 0.5 ml of 30 mM-Hepes (pH 8.0)/200 mM-sucrose/40 mM-NaCl/10 mM-MgCl₂/5 mM-EGTA/5 mM-ATP/0.2 mM-dATP/0.2 mM-dCTP/0.2 mM-dGTP/0.08 mM-[3 H]TTP (300–400 c.p.m./pmol) to the nuclei and incubating at 37 °C for 15 min. The reaction was terminated by adding 1 ml of ice-cold 20% trichloroacetic acid containing 0.1 M-sodium pyrophosphate. The nuclei were then held at 4 °C for 30 min before being washed with 2 $\times$ 1 ml of ice-cold 5% trichloroacetic acid containing 0.1 M-sodium pyrophosphate and once with 1 ml of ice-cold 95% ethanol. After drying, the nuclei were solubilized in 1 ml of 0.5% SDS. A 0.45 μ l sample was washed on to a glass-fibre filter as described [25] to determine incorporation of [3 H]TTP. Another 0.45 ml sample was used to determine DNA concentration [26].

Nuclear extracts

Nuclei were scraped from plates and pelleted by centrifugation at 800 *g* for 5 min at 4 °C. In accordance with a protocol of Eichler *et al.* [27], the pellet was resuspended in 9 vol. of 5 mM-KH₂PO₄ (pH 8.2)/2 mM-MgCl₂/1 mM-EDTA/1 mM- β -mercaptoethanol. The nuclei were disrupted in a Dounce homogenizer with a tight-fitting pestle. The concentration of the buffer was adjusted to 0.2 M by adding 1 M-KH₂PO₄, pH 8.2. The suspension was stirred for 60 min at 4 °C and then spun for 90 min at 100 000 *g*. The supernatant was made 20% in glycerol, frozen in liquid nitrogen and stored at –70 °C. The protein content of extracts was measured by the method of Bradford [28], with BSA as a standard.

DNA polymerase activity in extracts

DNA polymerase activity was assayed at 37 °C for 30 min in a 100 μ l assay mixture containing 50 mM-Tris/HCl (pH 8.0), 8 mM-MgCl₂, 0.5 mM-dithiothreitol, 0.05 mM each of dATP, dCTP, dGTP and [3 H]TTP (150–180 c.p.m./pmol), 0.3 mg of

gapped calf thymus DNA/ml and 0.3 mg of BSA/ml. The precipitation and washing of [3 H]DNA were as described elsewhere [25]. Incorporation was linear with added extract under the assay conditions.

Aphidicolin [29] and d₂TTP [30] were employed to determine the contribution of different DNA polymerases to the total activity. Extracts were assayed in the presence of increasing amounts of each inhibitor until no further decrease in activity was observed. Maximal inhibition was obtained at 8 μ M aphidicolin and 300 μ M-d₂TTP (results not shown). A combination of 8 μ M-aphidicolin and 300 μ M-d₂TTP decreased total DNA polymerase activity by more than 95%.

Dihydrofolate reductase (DHFR) and ornithine decarboxylase (ODC) assays

The preparation of cell lysates and quantification of DHFR by binding of [3 H]methotrexate were as described by Johnson *et al.* [31]. In order to detect only the accumulation of DHFR *de novo* after stimulation, cells were exposed to 1 μ M-methotrexate for 20 min before the end of amino acid starvation. ODC activity in cell lysates was measured as described by McConlogue *et al.* [32].

RESULTS

DNA replication in intact cells

As an essential reference point for our studies, we quantified the inhibition of DNA synthesis in quiescent cells stimulated to proliferate under conditions of mevalonate deprivation. After inducing quiescence by starvation for isoleucine and glutamine for 48 h, cells were stimulated to proliferate by returning them to complete medium. Synthesis of mevalonic acid was prevented by adding compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, at stimulation. As shown in Fig. 1(a), [3 H]thymidine incorporation during S phase was decreased by approx. 50% when cells starved for 48 h were stimulated in the presence of 10 μ M-compactin. In 18 experiments, incorporation at 21 h after stimulation was decreased by an average of 52%. Thymidine incorporation was equal to the control level when cells were stimulated in the presence of 10 μ M-compactin and 1 mM-mevalonate. Compactin treatment did not affect DNA replication uniformly in all cells. The fraction of 3 H-labelled nuclei detected by autoradiography during S phase was decreased by 30–50% relative to control cells (results not shown), approximately the same decrease observed for thymidine incorporation. This latter finding indicates that cells that do not synthesize any DNA are responsible for the net decrease in thymidine incorporation and is consistent with flow-cytometry studies that show that mevalonate-deprived cells have a G₁-phase DNA content [8,9]. Significantly, thymidine incorporation could not be further depressed by increasing the concentration of compactin to 40 μ M or by substituting 20 μ M-mevinolin [20], another inhibitor of HMG-CoA reductase (results not shown). The above observations show that DNA replication in approx. 50% of cells in the experimental population was refractory to inhibition of HMG-CoA reductase. These cells incorporated thymidine in the presence of high levels of inhibitors and were competent to synthesize DNA with little or no added mevalonate.

Importantly, DNA synthesis could be further depressed to approx. 20% of control levels when quiescence was induced by 72 h rather than 48 h of amino acid starvation (Fig. 1b). In 11 such experiments, thymidine incorporation was depressed by an average of 79% at 25 h after stimulation. Such a decrease in DNA synthesis is comparable with that observed when cells made quiescent by culture in plasma-derived lipoprotein-deficient serum were stimulated by platelet-derived growth factor in the presence of mevinolin [6,10]. The viability of our treated and

Fig. 1. $[^3\text{H}]$ Thymidine incorporation in cells starved for isoleucine and glutamine for 48 h (a) or 72 h (b) before return to complete medium containing no additions (○), 10 μM -compactin (●) or 10 μM -compactin and 1 mM-mevalonate (■).

untreated cultures was the same, as determined by Trypan Blue exclusion (results not shown), so a difference in mortality did not cause the increased inhibition. As in the previous experiments, adding mevalonate along with compactin restored thymidine incorporation though with less uniformity. In two of five experiments in which mevalonate was added with compactin, control levels of thymidine incorporation were found at every time point. In the other three experiments, a decrease of 6–35% was observed in 11 of 26 time points and showed no correlation with time after stimulation. The average restoration of thymidine incorporation in these three experiments was 84% and in all five experiments was 90%. Apparently, extended amino acid starvation under our experimental conditions can sporadically prevent the normal utilization of exogenous mevalonate in compactin-treated cells, or prevent thymidine incorporation by some other mechanism. Because the effect of compactin was amplified by extended quiescence, 72 h of amino acid starvation was used in subsequent experiments to demonstrate an enhanced effect and 48 h of starvation was used to ensure against sporadic deleterious consequences of prolonged amino acid deprivation.

Protein and RNA synthesis in intact cells

Inhibition of protein or RNA synthesis before S phase prevents DNA replication [33]. The pivotal role of mevalonate in several biosynthetic pathways suggests that the inhibition of DNA replication could be a secondary effect of mevalonate deficiency on the synthesis of other classes of macromolecules. However, our data demonstrate that general inhibition of RNA or protein synthesis before S phase is not the cause of the failure of DNA synthesis. As shown in Fig. 2, compactin did not significantly affect incorporation of ^3H -labelled amino acids at any time after

Fig. 2. Incorporation of $[^3\text{H}]$ amino acids in cells starved for isoleucine and glutamine for 48 h before return to complete medium containing no additions (○) or 10 μM -compactin (●).

Fig. 3. $[^3\text{H}]$ Uridine incorporation in cells starved for amino acids for 48 h (a) or 72 h (b) before stimulation with complete medium containing no additions (○), 10 μM -compactin (●) or 10 μM -compactin and 1 mM-mevalonate (■).

cells starved for 48 h were stimulated to proliferate, and the same was true for cells starved of amino acids for 72 h (results not shown). Likewise, as shown in Fig. 3(a), compactin did not affect incorporation of $[^3\text{H}]$ uridine in cells starved for 48 h. As shown in Fig. 3(b), after 72 h of starvation compactin did decrease uridine incorporation. The decrease increased from 5% at 7 h to 35% at 25 h (average of four experiments). Adding exogenous mevalonate restored uridine incorporation to, or near to, control levels. In two experiments in which mevalonate was added with compactin, a decrease of 0–22% was observed which showed no

correlation with time after stimulation. Of 12 time points, restoration was complete for 6 and averaged 85% for the remainder. Thus, as for thymidine incorporation, 72 h of amino acid starvation can sporadically prevent normal utilization of exogenous mevalonate in compactin-treated cells or inhibit uridine incorporation by some other mechanism.

DNA synthesis in isolated nuclei and nuclear extracts

The proximal causes of decreased DNA synthesis in compactin-treated cells may include diminished activity of DNA-replication enzymes, consistent with the observation that exponentially growing cells deprived of mevalonate are arrested in G₁ phase [8,9,34]. A different explanation is raised by the finding that mevalonate deprivation alters cellular morphology [35] and disrupts the integrity of the nuclear lamina [36]. DNA replication is dependent on cytoskeletal integrity, as demonstrated by the failure of quiescent cells to initiate DNA synthesis after stimulation in the presence of dihydrocytochalasin B, a drug that disrupts actin cytoarchitecture [37]. Alteration of the nuclear lamina may change chromatin structure so that DNA-synthesizing enzymes cannot assemble at replication origins and initiate synthesis. To evaluate these possibilities, we tested the competence of nuclei isolated from compactin-treated cells to carry out DNA synthesis. As shown in Fig. 4, TTP incorporation in nuclei of cells stimulated after 48 h of amino acid starvation resembled thymidine incorporation by whole cells (Fig. 1a). Like thymidine incorporation, TTP incorporation was decreased by 50% in nuclei of cells treated with 10 μ M-compactin. In 8 experiments, incorporation was decreased by an average of 54% at 21 h after stimulation. No decrease was observed in nuclei from cells treated concurrently with compactin and mevalonate. These results demonstrate that the compactin-induced decrease in nuclear DNA synthesis is identical with that of replication in whole cells. In addition, these findings eliminate the possibility that decreased DNA replication in mevalonate-deprived cells is due solely to (1) non-permissive pH, bivalent-cation concentrations or ionic strength in the nucleoplasm, (2) the presence of soluble inhibitors, or (3) the lack of deoxynucleoside triphosphates. These conditions would be made evident if the permeabilized nuclei prepared from compactin-treated cells were competent to synthesize DNA in our assay *in vitro*.

To evaluate whether decreased activity of DNA-replication enzymes or altered nuclear morphology contributes to diminished DNA replication in mevalonate-deprived cells, we assayed DNA polymerase activity in nuclear extracts. As shown in Fig. 5(a), extractable nuclear DNA polymerase activity in cells starved for amino acids for 48 h (Fig. 5a) or 72 h (Fig. 5b) increased during S phase. After 48 h of amino acid starvation (Fig. 5a), 10 μ M-compactin depressed the increase in DNA polymerase activity by an average of 59% at 24 h after stimulation in three experiments. This decrease is essentially the same as that found for thymidine incorporation in whole cells (Fig. 1a) and TTP incorporation in intact nuclei (Fig. 4). A similar decrease in TTP incorporation was observed with crude whole cell extracts, showing that DNA polymerase activity was not selectively lost when nuclei were prepared from compactin-treated cells (results not shown). Compactin treatment of cells starved for 72 h (Fig. 5b) completely abolished the S-phase increase in DNA polymerase activity, consistent with the greater inhibition of thymidine incorporation in these cells (Fig. 1b). Concurrent treatment with compactin and 1 mM-mevalonate completely restored DNA polymerase activity. These findings demonstrate that the increase in DNA polymerase activity associated with replication is dependent on mevalonic acid. They also show that the decreased thymidine incorporation in whole cells can be completely accounted for by the decrease in DNA polymerase activity. This decrease does not exclude other

Fig. 4. [³H]TTP incorporation in nuclei of cells deprived of isoleucine and glutamine for 48 h before stimulation in complete medium containing no additions (○), 10 μ M-compactin (●) or 10 μ M-compactin and 1 mM-mevalonate (■).

Fig. 5. DNA polymerase activity extracted from nuclei of cells starved for isoleucine and glutamine for 48 h (a) or 72 h (b) before return to complete medium containing no additions (○), compactin (●) or compactin and 1 mM-mevalonate (■).

Compactin concentration was 10 μ M in (a) and 40 μ M in (b).

causes of the deficit in replication. Such causes may co-exist in mevalonate-deprived cells, but they do not abolish the activity of otherwise competent DNA polymerase.

Effect of length of amino acid deprivation on DNA replication

Our results indicate that, as quiescence continues, an expanding fraction of cells loses the ability to synthesize DNA without a source of mevalonate (Figs. 1 and 5). To confirm this observation,

Table 1. Effect of length of isoleucine and glutamine deprivation on DNA replication

Values for 48 h and 72 h amino acid deprivation are from Figs. 1 and 5. They are determined for incorporation 24 h after stimulation. Values for 36 h deprivation were determined at the peak of thymidine incorporation 16 h after stimulation.

Period of amino acid deprivation	Decrease in incorporation (%)	
	Thymidine	TTP
36 h	41	41
48 h	51	59
72 h	79	100

Table 2. Effect of aphidicolin and d₂TTP on DNA polymerase activity of cells stimulated after 48 h of amino acid starvation

Nuclear extracts were prepared 24 h after stimulation. Cells were treated with 10 μ M-compactin. Activity was determined in the absence and presence of either aphidicolin or d₂TTP. Total activity and resistant activity were measured directly, and sensitive activity was calculated as the difference. Adding 40 μ M-compactin or 1 mM-mevalonate to DNA polymerase assays containing nuclear extracts or fractionated DNA polymerase α or β had no effect on activity.

	Activity (pmol of [³ H]TTP/ μ g of DNA)		
	No inhibitor	Aphidicolin-resistant	Aphidicolin-sensitive
Expt. 1			
Control	1.77	0.25	1.52
Compactin	0.58	0.22	0.36
	No inhibitor	d ₂ TTP-resistant	d ₂ TTP-sensitive
Expt. 2			
Control	1.43	1.30	0.14
Compactin	0.6	0.44	0.18

Table 3. Effect of compactin on the increase in DHFR 24 h after stimulation

Cells were deprived of amino acids for 48 h before extracts were prepared at 0 and 24 h after stimulation. The amount of DHFR was measured as pmol of [³H]methotrexate bound/ μ g of protein, and the relative increase between 0 and 24 h was calculated for control and compactin-treated cells.

Expt. no.	Relative increase		
	Control	Compactin	Compactin/control
1	3.2	2.5	0.78
2	1.6	1.6	1.00
3	1.8	1.6	0.89
4	2.4	1.6	0.64
5	5.5	5.8	1.05
Mean $\pm$ S.E. = 0.87 $\pm$ 0.15			

we determined the effect of compactin on DNA synthesis in cells stimulated to proliferate after 36 h of isoleucine and glutamine deprivation. As shown in Table 1, the incorporation of thymidine in whole cells and TTP by a nuclear extract is decreased to a

similar extent. More importantly, Table 1 confirms that lengthening the period of isoleucine and glutamine deprivation diminishes both thymidine incorporation and DNA polymerase activity. The fact that nuclei from cells treated with compactin for 72 h lose all capacity to incorporate TTP indicates that the entire population is susceptible to the drug. The progressive and nearly total loss of competency to replicate DNA by compactin-treated cells suggests that one or more mevalonate-dependent factors required for DNA replication are depleted during quiescence induced by amino acid deprivation.

Activity of replicative DNA polymerases

DNA polymerase α , and likely polymerases δ and ϵ , are required for mammalian DNA replication [38]. All three polymerases, like replication itself, are inhibited by aphidicolin [39,40]. In contrast, DNA polymerase β , another nuclear DNA polymerase which functions primarily in DNA repair [38], is insensitive to aphidicolin, but is inhibited by d₂TTP [30]. We used the differential sensitivity to aphidicolin and d₂TTP to determine whether mevalonate insufficiency selectively affects replicative DNA polymerases. As shown in Table 2, in the absence of either of these inhibitors, compactin decreases total DNA polymerase activity by approx. 60% (Expts. 1 and 2, column 1). In Expt. 1, DNA polymerase activity extracted from nuclei was assayed in the presence of aphidicolin (columns 2 and 3). The component of the total activity resistant to aphidicolin (column 2), i.e. repair DNA polymerase β , was insensitive to compactin. The component of total activity that is aphidicolin-sensitive (column 3), however, was decreased by compactin. Thus compactin selectively affected replicative DNA polymerase activity. This conclusion was supported by the results of Expt. 2, which used the inhibitor d₂TTP (columns 2 and 3). The d₂TTP-sensitive component of activity (column 3), DNA polymerase β , was not affected by compactin. The d₂TTP-resistant component (column 2) i.e. replicative DNA polymerases α , δ and ϵ , was diminished by compactin. The results of both of these experiments demonstrated that only replicative DNA polymerase is mevalonate-dependent.

DHFR level

To determine if other enzymes involved in DNA replication are affected by mevalonate deficiency, we assessed the effect of compactin on DHFR. DHFR is required for purine and thymidylate synthesis, and its inhibition blocks DNA replication and halts proliferation [41]. The amount and activity of DHFR increases during S phase after quiescent cells are stimulated to proliferate [41]. As shown in Table 3, the increase in DHFR in compactin-treated cells 24 h after stimulation was only 13% less than that in control cells. In the same five experiments, the average increase in DHFR in compactin-treated cells was the same as in controls at 6, 12, 15 and 18 h after stimulation (results not shown). Additionally, in a sixth experiment measurement of DHFR activity showed no consistent decrease by compactin during S phase, the ratio of activities of compactin-treated to control cells being 1.0, 1.2, 0.80, 1.2 and 0.89 at 12, 16, 18, 20 and 22 h after stimulation respectively. These results show that at least one enzyme required for DNA replication is not significantly affected by mevalonate deprivation.

ODC activity

Although rates of total protein and RNA synthesis before S phase were unaffected by compactin, these parameters would not necessarily reflect subtle or limited metabolic changes which preclude DNA replication. To monitor a more sensitive indicator of proliferative state, we assayed ODC activity, which rises and falls rapidly in response to a variety of positive and negative growth effectors [42]. As shown in Fig. 6, ODC activity rose after

Fig. 6. Activity of ODC in cells deprived of isoleucine and glutamine for 48 h before stimulation with complete medium containing no additions (○) or 10 μ M-compactin (●)

The assay measures the conversion of [14 C]ornithine into putrescine and 14 CO $_2$. Mean values were calculated from duplicate determinations which differed by less than 10%.

Fig. 7. [3 H]Thymidine incorporation in cells starved for amino acids for 48 h before return to complete medium containing no additions (○), 10 mM-putrescine (■), 10 μ M-compactin (●) or 10 μ M-compactin and 10 mM-putrescine (□)

quiescent cells were stimulated and remained high throughout S phase, as is characteristic of HTC cells [43]. In the presence of compactin, ODC activity was detectably lower 6 h after stimulation and remained decreased thereafter. In three experiments the average ODC activity in compactin-treated cells decreased from 78% of control at 6 h after stimulation to 23% of control at 24 h after stimulation (results not shown). Notably, the decrease in ODC activity in the three experiments occurred at least 6 h before the onset of S phase.

Putrescine, the product of ODC, is the precursor of other polyamines believed essential for DNA replication [44]. Exogenous putrescine restores DNA replication in cells whose ODC activity is inhibited by drugs [32,45] or eliminated by mutation [46]. However, as shown in Fig. 7, exogenous putrescine had no effect on the decreased thymidine incorporation by compactin-treated cells. These results demonstrate that ODC activity is dependent on mevalonic acid and show, in addition, that DNA replication cannot be restored in mevalonate-starved cells by supplying the product of ODC activity.

DISCUSSION

Since the initial description by this laboratory of the mevalonate-dependence of DNA replication [3], numerous papers have described the consequences of mevalonate insufficiency on growth, cell cycle progression and morphology [4,6–10,47]. However, the detailed mechanisms by which mevalonate deprivation blocks DNA synthesis are not understood. The identity of the non-sterol derivative(s) of mevalonate required for growth remains elusive, and the role of prenyl-modified proteins in DNA replication and cellular proliferation remain to be defined. To begin to define the biochemical basis of the block to DNA replication, we examined enzymes required for DNA replication in rat hepatoma cells emerging from quiescence in the absence of mevalonate synthesis.

We found that a decrease in aphidicolin-sensitive DNA polymerase activity is sufficient to account completely for the deficit of DNA replication in mevalonate-deprived cells. Ours is the first direct demonstration of this mechanism. We considered other possible causes of the deficit. For example, DNA replication is dependent on cytoskeletal integrity [41], whereas mevalonate insufficiency disrupts nuclear [40] and cellular [39] morphology. Altered morphology could produce conformational changes in chromatin that prevent competent enzymes from initiating DNA replication. Also, morphological changes could disrupt transport through the nuclear membrane, critically altering the concentration of diverse factors either required for or inhibitory to DNA synthesis. However, our results exclude the possibility that failure to replicate DNA is solely the consequence of mevalonate insufficiency abolishing requisite morphological conditions for activity of competent DNA polymerase. Our findings do not eliminate additional causes for the failure to replicate DNA, but they do ensure that such causes do not mask a normal complement of DNA polymerase activity.

In addition, we observed that other enzymes required for DNA replication are not affected by mevalonate insufficiency. The level of DHFR, required for purine and thymidylate synthesis [41], was not affected in compactin-treated cells. The broader role that DHFR plays in cellular metabolism by providing C $_1$ units for the synthesis of amino acids [41] may exempt it from a mevalonate requirement. This suggests that mevalonate dependency may be characteristic of enzymes dedicated to DNA synthesis during the S phase. This hypothesis is supported by a report of depressed thymidine kinase activity in Swiss 3T3 cells emerging from quiescence in the absence of mevalonate synthesis [48]. Additional support comes from our finding that compactin has no effect on DNA polymerase β , which functions primarily in DNA repair rather than replication [38].

Importantly, the effect of mevalonate insufficiency on DNA replication manifests itself well before the onset of S phase. In light of the rapid decline in ODC activity when growth is slowed or stopped, the depression of this activity detected 6 h after stimulation probably reflects a mevalonate dependency of prior events necessary for transition from a quiescent to a proliferating state. The disruption by mevalonate starvation of one or more early steps in the ordered, as yet unelucidated, sequence of events required for DNA replication [33,49] after stimulation is consistent with the finding that exponentially growing cells deprived of mevalonate are arrested in G $_1$ [8,9,34]. It is also consistent with decreased accumulation of *c-fos* mRNA after stimulation of resting fibroblasts in the presence of lovastatin [50]. On the basis of these findings, we conclude that decreased replicative DNA polymerase activity in compactin-treated cells is likely to be an end-stage consequence of failure to execute prior requisite events.

Notably, the competency to replicate DNA without a source of mevalonate is progressively lost with increasing duration of

isoleucine and glutamine deprivation. This result suggests that cells arrested by amino acid starvation are gradually depleted of one or more mevalonate-dependent factors required for DNA replication. The loss of mevalonate-derived products during quiescence is supported by the observation of decreased HMG-CoA reductase activity and decreased synthesis of known mevalonate derivatives in resting cells [7], including cells arrested by isoleucine deprivation [51]. Presumably, these factors are restored shortly after stimulation of cells capable of synthesizing mevalonate.

The identity of the non-sterol products of mevalonate required for DNA replication and proliferation are not known. The characterization of proteins modified by prenyl derivatives may provide mechanistic insights into the mevalonate-dependency of DNA replication and cell-cycle progression. Prenylated proteins are not maintained when exponentially growing cells are deprived of mevalonate [47], and their rate of disappearance closely correlates with the rate of loss of capacity to replicate DNA [8]. By analogy, depletion during quiescence of prenylated proteins which are required for the transition to a proliferative state, coupled with the inability to perform synthesis *de novo* of prenyl ligands, may be ultimately responsible for the failure of compactin-treated cells to replicate DNA in our experiments.

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