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Cell Cycle Arrest in Archaea by the Hypusination Inhibitor N^1-Guanyl-1,7-Diaminoheptane

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Hypusination is an essential posttranslational modification unique to archaeal and eukaryotic protein synthesis initiation factor 5A (aIF5A and eIF5A, respectively). We have investigated the effect of the efficient hypusination inhibitor N^1-guanyl-1,7-diaminoheptane (GC_7) on four archaeal and one bacterial species. We found that (i) archaea are sensitive to GC_7, whereas the bacterium *Escherichia coli* is not, (ii) GC_7 causes rapid and reversible arrest of growth of the archaeon *Sulfolobus acidocaldarius*, and (iii) the growth arrest is accompanied by a specific reversible arrest of the cell cycle prior to cell division. Our findings establish a link between hypusination and sustained growth of archaea and thereby provide the framework to study molecular details of archaeal cell cycle in connection with in vivo functions of hypusine and of aIF5A and eIF5A.

Eukaryotic translation initiation factor 5A (eIF5A) stimulates ribosomal peptidyltransferase. The biochemical activity of eIF5A, as well as sustained growth of eukaryotic cells, requires hypusine [N^1-(4-amino-2-hydroxybutyl)lysine], a posttranslationally modified amino acid unique to eukaryotic and archaeal IF5A (eIF5A and aIF5A, respectively) (see reference 10 for a recent review). The function of aIF5A is inferred from that of eIF5A, since no reliable cell-free translation systems from archaea have been established. Hypusination of pre-IF5A proceeds in two steps. First, deoxyhypusine synthase (DHS) transfers the aminobutyryl moiety of the polyamine spermidine to the ε-amine of a conserved lysine in pre-IF5A to form deoxyhypusine. Next, deoxyhypusine hydroxylase adds a hydroxyl group to the η-carbon to form hypusine. Deoxyhypusination occurred in all eukaryotes (4) and archaea (1) tested. N^1-guanyl-1,7-diaminoheptane (GC_7) is a very efficient inhibitor of DHS in eukaryotes. This homolog of spermidine inhibits the first step in the hypusination pathway by binding to DHS, occluding the binding site for spermidine, with an apparent K_i of 10 μM (6, 9). Hypusine, the product of the second reaction, has been found in all eukaryotes and crenarchaea tested, whereas euryarchaea seem not to perform the second modification step (1). Neither deoxyhypusine nor hypusine have been found in bacteria (4). The current model of eIF5A's involvement in protein synthesis and cell proliferation suggests that it is required for correct translation of a subset of mRNAs, possibly including ones that encode key proteins for cell growth and proliferation (7).

Members of the genus *Sulfolobus* are the first archaea whose cell cycle features have been determined (2). The cell cycle is characterized by a short prereplicative period (B or G1 period; 1 chromosome eq/cell) and a long postreplicative period (D or G2 period; 2 chromosome eq/cell). Another unusual feature is the presence of 2 chromosome eq/cell in stationary phase.

We have investigated the effects of the highly efficient pharmacological inhibitor of DHS, GC_7 (6), on the growth of four archaeal and one bacterial species. We have also analyzed, by flow cytometry, the detailed effects on the cell cycle of *Sulfolobus acidocaldarius*.

Growth inhibition by GC_7. Four different species of archaea and one bacterium were tested for their sensitivity to GC_7. The antibiotic was synthesized and separated as previously described (3), dissolved to 0.05 M in 1.0 mM acetic acid, and kept frozen. *S. acidocaldarius* DSM 639 and *Sulfolobus solfataricus* DSM 1616 were grown as previously described (2). *Halobacterium halobium* DSM 670 and *Haloferax mediterranei* DSM 1411 were grown at 37°C (11) (the low-salt medium described in reference 11 for *Haloferax volcanii* was used to grow *H. mediterranei*). *Escherichia coli* MG 1655 was grown at 37°C in Luria-Bertani medium. All species were grown in liquid medium in water baths at a shaking speed of 200 rpm. Aliquots taken from exponentially growing cultures were used to inoculate 25 ml of preheated liquid media. The cells were then allowed to grow one generation prior to addition of GC_7 to final concentrations of 100 and 1,000 μM. Growth was monitored by measuring the optical density at 600 nm (OD_{600}).

Archaeal growth was slightly affected by the presence of a low concentration of GC_7 (100 μM) (except a strong growth inhibition in the case of *S. acidocaldarius*) (Table 1) and totally arrested at a higher concentration (1,000 μM). Aminoguanidine was not required to enhance the effect of GC_7, in contrast to eukaryotes (9). At the higher concentration, only a slight decrease in the *E. coli* growth rate could be observed. Hence, GC_7 is effective as an antibiotic on all archaea tested but not on the bacterium *E. coli*. The relative insensitivity of *E. coli* to GC_7 is consistent with the following. (i) Bacteria lack genes with significant homology to DHS genes from either eukaryotes or archaea. (ii) The homolog of aIF5A or eIF5A in bacteria, elongation factor P (8), contains a posttranslational modification other than hypusine or deoxyhypusine (H. E. Johansson et al., unpublished). The slight effect of high drug concentrations on bacterial growth could instead be due to unspecific effects of the increased polyamine level.

Finally, in preliminary experiments using phase-contrast and fluorescence microscopy, no change in either cell or nucleoid morphology could be observed during GC_7 treatment. GC_7 thus seems not to allow further expansion of cell mass or induce DNA degradation. We conclude that GC_7 causes growth arrest of deoxyhypusine-synthesizing archaea, an arrest that in

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TABLE 1. Inhibition of archaeal and bacterial growth by GC_7[a]

Species	Growth at 100 μM GC_7	Growth at 1,000 μM GC_7
S. acidocaldarius	+	−
S. solfataricus	+++	−
H. halobium	+++	−
H. mediterranei	+++	−
E. coli	+++	++

[a] +++, normal growth; ++, slight increase in doubling time (1.3); +, doubling time increased by a factor of 2; −, no measurable growth. See text for details on conditions and monitoring of growth.

S. acidocaldarius could be reflected in a specific arrest of the cell cycle.

Dose-dependent inhibition of *S. acidocaldarius* growth. Three features of the archaeon *S. acidocaldarius* made it suitable to study the effect of GC_7 in more detail. (i) Its cell cycle has been characterized (2). (ii) It lends itself well to flow cytometric analyses. (iii) Its aIF5A protein has been isolated and shown to be fully hypusinated (12). *S. acidocaldarius* was grown in the presence of 0, 50, 100, 200, 350, 500, or 1,000 μM GC_7 to determine the concentration necessary to affect growth. The growth curves presented in Fig. 1A show that the growth rate was inversely proportional to the concentration of GC_7. The effect is thus cumulative and increases until growth ceases completely. We used the slope of the linear part of the growth curve in a logarithmic plot as an approximate measure of the growth rates. From these, the dose of drug at which the growth rate was reduced by 50% was deduced to be about 90 μM (data not shown). This concentration is similar to that required for growth arrest of eukaryotic cells (30 to 100 μM) (3, 13). The continued analysis of the effect of GC_7 on the cell cycle requires inhibited growth over an extended period (>24 h) without major morphological changes to the cells. We chose a concentration of 200 μM at which growth is inhibited sufficiently (Fig. 1A), while the cells retain normal cell and nucleoid shape as judged by phase-contrast and fluorescence microscopy (data not shown).

GC_7 causes cell cycle arrest. We examined the growth arrest in more detail. GC_7 was added to exponentially growing *S. acidocaldarius* to a final concentration of 200 μM. Measurements of OD_{600} every hour after drug addition were used to monitor growth and confirmed the growth arrest (the OD_{600} changed only from 0.2 at the time of drug addition to 0.23 5 h later, while in the untreated culture the OD_{600} increased from 0.13 to 0.35).

To determine the effect of GC_7 on the *S. acidocaldarius* cell cycle, samples were taken every 15 min and fixed in ethanol for subsequent analysis by flow cytometry (for details of the method, see reference 2). Flow cytometric analysis gave two sets of data for each sample: light scatter, which roughly represents cell size, and fluorescence, which is proportional to the DNA content of each cell analyzed. GC_7 had no effect on cell size during the 5 h of this analysis (Fig. 1B). In contrast, a rapid effect on the DNA distribution was observed: the cell population with 1 chromosome eq/cell decreased within 15 min after drug addition until it had completely disappeared 2 h after addition. Within the time frame of one cell cycle (3.5 h), all cells had arrested at the two-genome stage. This indicates that GC_7 provokes cell cycle arrest in the D (G2) phase and that it does not prevent the elongation phase of DNA replication. At a higher GC_7 concentration (500 μM) (data not shown), replication was slowed down, but the cellular response to the drug was essentially the same: the cells arrested in the postreplication stage.

There are two noticeable differences in the response to GC_7 by *S. acidocaldarius*, compared to eukaryotic cells. First, the archaeon responded quickly to the drug (the cells stop dividing within 15 min after drug addition), while two to four cell cycles have to be completed in eukaryotes before arrest (13). This may be due to a higher turnover rate of aIF5A than of eIF5A, which is unusually long in yeast (48 h) (7). However, targets for GC_7 other than DHS cannot be excluded. The second difference lies in the timing of the arrest during the cell cycle: the end of the D period in *Sulfolobus* and the G1-S transition in eukaryotes. The proportion of the cell population in the B (G1) period has been estimated to be about 5% in exponentially growing *S. acidocaldarius* cultures (2). However, this population disappeared after the drug treatment, suggesting that replication initiation itself (the B-C or G1-S transition) was not blocked. Further work must be done to clarify some of the underlying reasons for these clear differences.

Reversion of GC_7-induced growth inhibition. The usefulness of GC_7 to study the molecular details of hypusination in archaea would be greatly enhanced if cell growth could be restored after withdrawal of the inhibitor. We therefore performed an experiment on *S. acidocaldarius* where the inhibitor (200 μM) was removed or reduced to a noneffective concentration after 4 h of treatment, a time sufficient to cause cessation of growth and complete cell cycle arrest (Fig. 1C, graph i). Reduction of the GC_7 concentration was achieved by two methods: (i) centrifugation (10 min at 12000 × *g*) followed by resuspension of the cells in preheated drug-free medium or (ii) a fivefold dilution of the culture. Samples were taken at 2-h intervals before the release from growth arrest induced by GC_7 (for OD measurements) and after the release at 1-h intervals (for OD measurements) or at 15-min intervals (for flow cytometry). Samples were prepared as described above. Both treated cultures quickly resumed growth after reduction of the GC_7 content by either method (Fig. 1C, panel i). Both untreated cultures and the treated culture after removal of GC_7 by centrifugation grew slightly faster than the diluted culture, presumably due to residual GC_7 (approximately 40 μM) (compare Fig. 1C, panel i, with Fig. 1A). The effect of GC_7 of *S. acidocaldarius* thus appeared to be cytostatic.

Flow cytometric analysis of the diluted culture showed that the cells resumed division and replication within 120 min (Fig. 1C, panel ii). This was manifested in a decrease in light scatter after 105 min with a concomitant increase of the one-chromosome peak. A slight increase in cell size, as indicated by an increase in light scatter between 0 and 60 min, could be an effect of dilution as seen in experiments by others (5). The partial synchrony of the cell cycle achieved after removal of GC_7 opens up possibilities for its use as a synchronizing agent in cell cycle studies.

The entry into and release from growth arrest induced by GC_7 was faster than that for cells that enter and leave stationary phase (data not shown) (5). The flow cytometric patterns of cells that resumed growth underscore the faster recovery after release from GC_7. The cells restarting growth from stationary phase also display a larger variation in light scatter until they resume cell division, compared to the cells released from GC_7-induced growth arrest.

Conclusions. We have analyzed how the hypusination inhibitor GC_7 affects growth of four different archaea and the bacterium *E. coli*. The drug was found to be effective on all archaea tested, at concentrations comparable to those used in similar studies on eukaryotes, but to be ineffective on the bacterium. Arrest of both cellular growth and the cell cycle of the hypusine-containing archaeon *S. acidocaldarius* was rapid and reversible. The rapidity of the effect is in strong contrast to the slow effect seen on mammalian cells, while reversibility for

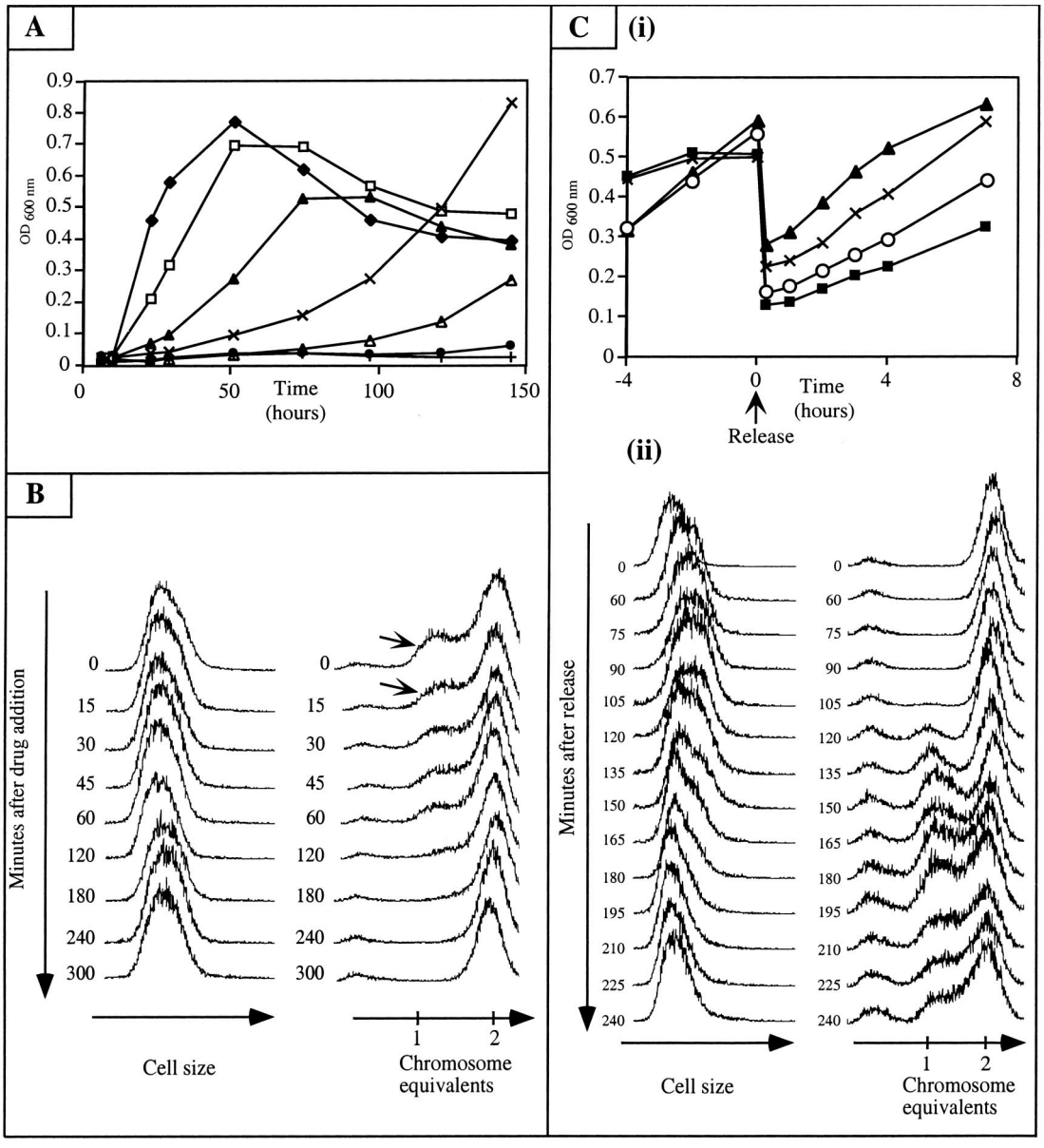

FIG. 1. (A) Dose-dependent inhibition of *S. acidocaldarius* growth by GC_7. *S. acidocaldarius* was subjected to 0 (◆), 50 (□), 100 (▲), 200 (×), 350 (△), 500 (●), and 1,000 (+) μM concentrations of GC_7. Growth was monitored by OD_{600} measurements at the indicated time points. The growth curves give approximate doubling times of 3.5 h (0 μM GC_7), 4.5 h (50), 8.0 h (100), 20 h (200), 25 h (350), and 50 h (500), whereas no growth could be detected at 1,000 μM GC_7. (B) Analysis of GC_7 effects on the *S. acidocaldarius* cell cycle. Samples were taken every 15 min after addition of GC_7 (200 μM), fixed in ethanol, stained with mithramycin and ethidium bromide, and analyzed by flow cytometry. The left column represents the light scatter (cell size), and the right column represents the fluorescence (DNA content) of the cell population from the same samples. (C) The effect by GC_7 is cytostatic. *S. acidocaldarius* resumed normal cell growth (i), including cell division and replication (ii), after reduction of the GC_7 concentration to noneffective levels. *S. acidocaldarius* cells were treated with 200 μM GC_7 or left untreated, and the drug was removed after 4 h. The release was accomplished either by dilution (○ and ■) or by change of medium after centrifugation (▲ and ×). (i) Growth curves were determined by OD_{600} measurements. The treated cultures (■ and ×) were compared to untreated cultures (○ and ▲). (ii) Flow cytometric analysis of samples fixed in ethanol every 15 min after release by dilution (samples correspond to treated culture dilution curve [■] in graph i).

eukaryotes has not been reported. The effect of GC_7 on the cell cycle of *S. acidocaldarius* included arrest at the end of the D period (G2) (prior to cell division), without an apparent effect on replication elongation.

The rather well-defined arrest of the cell cycle induced by GC_7 could be due to the requirement of functional aIF5A for the proper synthesis of a small number of proteins directly involved in cell cycle progression. It is also conceivable that GC_7 has a general effect on protein synthesis and that *S. acidocaldarius* uses the end of the D period of the cell cycle as a common checkpoint of cellular functions, including protein synthesis, before commencing chromosome segregation (mitosis) or actual cell division. At this point we cannot distinguish between the two possibilities. However, arrest of the cell cycle in the D period is common in several archaeal species treated with various antibiotics (K. Hjort, L. Malandrin, and R. Bernander, unpublished).

The presented results provide a framework to further delineate at which step the cell cycle arrests and to define which cellular process(es) is affected by the GC_7 treatment. To this

end, analysis of how the proteome changes in response to drug or starvation treatments will be essential. Here the sequence of the *S. solfataricus* genome, currently being worked out, should prove invaluable and allow us to better understand differences in the response to growth perturbations by members of the *Sulfolobus* genus. The continued investigations will also include molecular characterization of aIF5A (cellular content of aIF5A and of hypusine and deoxyhypusine) during the cell cycle.

In summary, we have established a simple and robust in vivo model system to study molecular details of the archaeal cell cycle and the in vivo function of hypusine and aIF5A.

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