In vivo inhibition of cyclin B degradation and induction of cell-cycle arrest in mammalian cells by the neutral cysteine protease inhibitor N-acetylleucylleucylnorleucinal (cell-cycle progression/mammalian cells)

Steven W. Sherwood*, Andrew L. Kung, Joseph Roitelman, Robert D. Simoni, and Robert T. Schimke

Department of Biological Sciences, Stanford University, Stanford, CA 94305

Contributed by Robert T. Schimke, December 14, 1992

ABSTRACT The cytotoxic neutral cysteine protease inhibitor N-acetylleucylleucylnorleucinal (ALLN) inhibits cell-cycle progression in CHO cells, affecting the G1/S and metaphase-anaphase transition points, as well as S phase. Mitotic arrest induced by ALLN is associated with the inhibition of cyclin B degradation. At mitosis-inhibiting concentrations of ALLN, cells undergo nuclear-envelope breakdown, spindle formation, chromosome condensation, and congression to the metaphase plate. However, normal anaphase events do not occur, and cells arrest in a metaphase configuration for a prolonged period. Steady-state levels of cyclin B increase to greater than normal mitotic levels, and cyclin B is not degraded for an extended period. Histone H1 kinase activity remains elevated during mitotic arrest. Duration of mitotic arrest depends on ALLN concentration; high concentrations (>50 μg/ml) produce a prolonged mitotic arrest, whereas at lower concentrations, cells are transiently delayed through mitosis (up to 4–12 hr), after which they undergo aberrant cell division resulting in randomly sized daughter cells containing variable amounts of DNA. Cyclin B degradation fails to occur, and histone H1 kinase remains activated for the duration of mitotic arrest at all ALLN concentrations.

Proteolysis of cyclin proteins is crucial in the regulation of cell-cycle progression in a wide variety of cell types (1). Cyclin B degradation appears to involve ubiquitination (2–4), and although mutant cyclin B protein is conjugated with ubiquitin, it is not degraded (2, 5). The failure of cyclin B degradation blocks cell-cycle progression at the metaphase–anaphase transition in vitro (2, 5). In developing clam embryo cells, normal cell-cycle progression requires that cyclin B degradation occur within a narrow time window (6), and thus both the timing and extent of cyclin degradation are important in regulating progression through mitosis. In vitro studies with clam oocyte extracts show that a variety of agents, including some protease inhibitors, can alter the rate of cyclin B proteolysis (7). Although important aspects of cyclin B proteolysis are known, many details of the process in vivo remain largely undescribed (8).

The cell-permeant tripeptide neutral cysteine protease inhibitor N-acetylleucylleucylnorleucinal (ALLN) significantly reduces the in vivo rate of degradation of the enzyme hydroxymethylglutaryl-CoA reductase in Chinese hamster ovary (CHO) cells (9). ALLN is cytotoxic to CHO cells, and selection with ALLN leads to the development of drug resistance, resulting from the amplification of the MDR 1 gene (10) as well as overexpression of an aldo-keto reductase (21). However, the cellular effects of ALLN responsible for its cytotoxicity have not been defined. In this report we show that ALLN is a strong inhibitor of cell-cycle progression in CHO cells. It is active at two cell-cycle transition points, G1/S and mitosis, and also inhibits S-phase progression under some circumstances. In mitosis, ALLN inhibits the metaphase–anaphase transition, and this effect is correlated with inhibition of cyclin B degradation and prolonged activation of histone H1 kinase activity. Although CHO cells treated with ALLN at <40 μg/ml eventually divide, they do so without undergoing a normal anaphase, and the resultant daughter cells are random in size and DNA content. Both mitotic arrest and aberrant cell divisions induced by ALLN are cytotoxic.

MATERIALS AND METHODS

Cell Culture. CHO cells (CHO AA8) were obtained from Larry Thompson, Lawrence Livermore National Laboratory (11). HeLa S3 cells were obtained from the American Type Culture Collection (ATCC). Both cell lines were maintained in a minimal Eagle's medium/10% fetal bovine serum/5 mM glutamine/penicillin-streptomycin at 100 units and 100 μg/ml, respectively/20 mM Hepes buffer. Monolayer cultures were maintained in a humidified atmosphere (5% CO2) and passaged approximately twice weekly. Cells were free of mycoplasma contamination determined by fluorescence microscopy of Hoechst-stained cells. Clonogenic survival was determined by treating asynchronous populations with specified amounts of ALLN, thoroughly washing the cells in phosphate-buffered saline (PBS), and replating them in fresh medium. Colonies (>50 cells) were counted 6–12 days later. Cell synchronization was by the method of mitotic shake-off, as described (11).

Flow Cytometry. Cells were trypsinized, washed in PBS, and fixed with ethanol (final concentration 70%). DNA content was measured on a Coulter Epics 753 flow cytometer (Coulter) after staining with propidium iodide by standard methods.

Immunoﬂuorescence Analysis. Cells grown on coverslips were fixed in absolute methanol at −20°C for 20 min after washing with PBS. Cells were stained for 2–12 hr with a monoclonal anti-cyclin B1 antibody (clone GNS-1; Pharmingen, San Diego), which had been diluted 1:500 in PBS/0.5% bovine serum albumin. The coverslips were rinsed in PBS and incubated with rhodamine-labeled goat anti-mouse IgG (Cappel Laboratories) for 15 min. Cells were mounted in PBS/glycerol, 1:1 containing Hoechst 33242 at 0.25 μg/ml. Tubulin staining was done with an anti-tubulin monoclonal antibody (Sigma) by using similar conditions with the addition of saponin (0.2%) to the incubation buffers.

Abbreviation: ALLN, N-acetylleucylleucylnorleucinal.

*To whom reprint requests should be addressed.
Biochemical Assays. Histone H1 kinase activity was measured as described (11). Briefly, total cell lysates were prepared by freeze–thawing cell pellets frozen at specified time points in liquid nitrogen. Aliquots containing equal amounts of protein (assayed by Bradford assay; Bio-Rad) were incubated at 30°C in a reaction buffer containing 5 μCi of [γ-32P]ATP (Amersham) and histone H1 for 30 min. The reaction was stopped by addition of 2× loading (1× loading buffer is 12 mM Tris-HCl/5% glycerol/0.4% SDS/3 mM 2-mercaptoethanol) buffer, samples were boiled, and labeled histone was resolved by SDS/12% PAGE. Phosphorylated histone H1 was detected by autoradiography quantified by scanning densitometry (LKB Ultrascan Densitometer).

Cyclin B1 levels were measured by immunoblotting. Frozen cell pellets were thawed on ice and lysed in buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 5 mM EGTA, 100 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, ALLN at 10 μg/ml, antipain at 10 μg/ml, 100 μM leupeptin. One hundred micrograms of total cellular protein was loaded per lane, and proteins were resolved by SDS/PAGE and blotted onto nitrocellulose, as described (9). Cyclin B1 was detected with monoclonal antibody against human cyclin B1 (0.4–0.5 μg/ml in fresh blocking solution), and immune complexes were detected with the enhanced chemiluminescence system (ECL; Amersham). Cyclin band intensities were measured by scanning densitometry.

RESULTS

ALLN Cytotoxicity. We had shown (10) by clonogenic assay that continuous exposure to ALLN is cytotoxic to CHO cells with an LD50 of 2 μg/ml or 5.6 μM. With ALLN concentrations that produce cell-cycle effects, the survival of CHO and HeLa cells rapidly declines as a function of both concentration and duration of exposure (Fig. 1).

![Fig. 1. Cell survival of CHO and HeLa S3 cells after transient ALLN exposure. Cells were treated during asynchronous growth for various lengths of time and replated into fresh medium. Cell lines and concentrations are as follows: □, CHO (15 μg/ml); ●, CHO (30 μg/ml); ■, HeLa S3 (12.5 μg/ml); ●, HeLa S3 (25 μg/ml); ■, HeLa S3 (50 μg/ml).]

Fig. 2. DNA histograms of ALLN-treated CHO cells. Asynchronously growing cells were treated at concentrations (μg/ml) indicated in upper right of each panel for 12 hr before fixing and staining with propidium iodide. Position of G1 cells is indicated with closed arrow, and daughter cells resulting from an-anaphasic divisions are indicated by open arrows (2 × 10⁴ cells per histogram).

Cell-Cycle Effects of ALLN. To examine the cell-cycle effects of ALLN, asynchronously growing CHO cells were treated with different concentrations for 12 hr and fixed for determination of cellular DNA content by flow cytometry. Cell-cycle perturbations induced by ALLN are complex and dose-related. At low concentrations, 2.5 μg/ml, cells are blocked only in early S phase (Fig. 2). The point in S phase at which cells are blocked is progressively earlier as ALLN concentrations increase. At ≈25 μg/ml cells are unable to enter S phase and become blocked at the G1/S boundary (Fig. 2). At 5 μg of ALLN per ml, there is a small accumulation of 4C cells indicative of a reduced rate of transit through mitosis, and hypodiploid cells are apparent in histograms (Fig. 2). This result reflects the induction of multipolar spindles occurring when progression through mitosis is delayed (12, 13). When ALLN concentration exceeds ≈15
phase, cells before arrest. The required arrest is observed and extreme chromatin condensation, showing cells die (Fig. 2).

Under these conditions, cells proceed into mitosis with transit into and through S phase, cells treated with concentrations lower than those required for complete mitotic arrest eventually proceed past the arrest point. For ALLN-induced mitotic delay, cells undergo abnormal mitosis after the delayed M transit (see Fig. 2; see below).

Mitotic-Arrest Phenotype of ALLN-Treated Cells. The mechanism by which ALLN inhibits mitotic progression was studied in synchronized cells exposed to different ALLN concentrations lower than 2C. The mitotic effects of ALLN were examined in experiments in which ALLN is added 12 hr after shake-off when most (>80%) cells have progressed beyond early S phase (Fig. 3C). Under these conditions, cells proceed into mitosis before undergoing cell-cycle arrest. DNA histograms typically show a broad 4C peak or two 4C peaks, reflecting extreme chromatin condensation during ALLN-induced mitotic arrest (Fig. 3C). As with transit into and through S phase, cells treated with concentrations lower than those required for complete mitotic arrest eventually proceed past the arrest point. For ALLN-induced mitotic delay, cells

μg/ml, cells with 4C DNA content accumulate, and there are no hypodiploid cells, indicating mitotic arrest (Fig. 2, see below). A slight decrease in the fluorescence of 4C cells is frequently observed and believed to reflect "hypercondensation" of chromosomes during metaphase arrest (Figs. 2 and 3). Similar cell-cycle effects were observed with HeLa S3 cells (data not shown).  

Cell-Cycle Arrest in Synchronized Cells. Cell-cycle arrest points were further examined by exposing synchronized CHO cells to ALLN at different times after mitosis. Untreated cells pass through mitosis 15 hr post-shake-off, and by 16 hr, most have returned to G1 (Fig. 3A). Addition of ALLN (40 μg/ml) 4 hr after mitosis, while cells are in G1, results in cell-cycle arrest at the G1/S boundary (Fig. 3B). Cells with <G1 DNA content at 18–24 hr after ALLN addition are apoptotic by morphological criteria (data not shown). At lower concentrations, cells progress further into S phase before arrest (Fig. 2), but even at concentrations as low as 2.5 μg/ml, relatively few cells continue normal cycling, and many S-phase-arrested cells die (data not shown).

The mitotic effects of ALLN were examined in experiments in which ALLN is added 12 hr after shake-off when most (>80%) cells have progressed beyond early S phase (Fig. 3C). Under these conditions, cells proceed into mitosis before undergoing cell-cycle arrest. DNA histograms typically show a broad 4C peak or two 4C peaks, reflecting extreme chromatin condensation during ALLN-induced mitotic arrest (Fig. 3C). As with transit into and through S phase, cells treated with concentrations lower than those required for complete mitotic arrest eventually proceed past the arrest point. For ALLN-induced mitotic delay, cells undergo abnormal mitosis after the delayed M transit (see Fig. 2; see below).
concentrations beginning 12 hr after mitosis. In cells treated with adequate ALLN to arrest mitosis, cells arrest with fully condensed chromosomes arrayed on the metaphase plate. Immunofluorescent staining with antitubulin antibody shows that interphase microtubules are not obviously affected by ALLN (data not shown), and normal-appearing metaphase spindles are initially formed in the presence of ALLN (Fig. 4A). After ~4–6 hr of metaphase arrest, however, spindle organization becomes abnormal due to disruption of spindle fibers and generation of multipolar spindles, as shown in Fig. 4A. The division of these cells occurs by random “pinching-off” of segments of cytoplasm containing variable numbers of chromosomes. Fig. 4B shows anti-tubulin staining of such a division, and 4C and D show the phase-contrast and Hoechst-stained images of a different cell undergoing this process. Because normal anaphase events do not occur during this division, we term this form of cell division “an-anaphasic.” An-anaphasic division generates the hypodiploid cells apparent in the DNA histograms described above (Fig. 2). Anti-cyclin staining indicates that cyclin B degradation occurs during an-anaphasic division (Fig. 4G and see below).

Effects of ALLN on Cyclin B Levels and Histone H1 Kinase Activity. The mitotic-arrest phenotype and the occurrence of an-anaphasic division in ALLN-treated cells are consistent with the possibility that ALLN alters the rate of cyclin degradation. The data in Fig. 5 show the results of experiments that examine cyclin B degradation and histone H1 kinase activity in synchronized CHO cells exposed to ALLN at 40 µg/ml 12 hr after mitosis. In untreated cells, the level of cyclin B rises rapidly to a sharp peak at 14 hr after mitosis (Fig. 5A and D). In treated cells, cyclin B accumulates slightly faster and attains maximal levels 1.6-fold higher than the maximal level measured in untreated cells (Fig. 5A and D). At this concentration, ALLN induces a prolonged mitotic arrest, and the cyclin level continues to increase up to 26 hr after mitosis (Fig. 5A and C). At lower ALLN concentrations, mitotic arrest is transient, and cyclin B levels decline to interphase levels at the time of an-anaphasic division. The timing and rate of cyclin degradation is inversely related to ALLN concentration. The same blots were also probed with anti-calpain II antibodies, which shows that calpain II levels are constant throughout the cell cycle in both control and ALLN-treated cells (Fig. 5B).

We also assayed histone H1 kinase activity in cell extracts prepared from synchronized cells exposed to ALLN 12 hr after mitosis. As with cyclin B levels, histone kinase activity increases beginning about 12 hr in both treated and untreated cells. Although kinase activity declines rapidly in untreated cells (peak kinase activity 14 hr after synchronization), ALLN causes H1 kinase activity to remain elevated for at least 24 hr after synchronization (Fig. 5D). At lower ALLN concentrations, H1 kinase activity begins to decline earlier. In general, the time at which the activity begins to decline as well as the rate of cyclin degradation are inversely related to ALLN concentration (data not shown). Thus, H1 kinase activity, like cyclin B levels, remains elevated for the duration of ALLN-induced mitotic arrest.

**DISCUSSION**

*In vitro,* ALLN partially inhibits cyclin B degradation in clam oocyte extracts (7). ALLN is cell permeant and reduces the *in vivo* rate of degradation of the enzyme hydroxymethylglutaryl-CoA reductase (9). In this report we show that ALLN is a potent *in vivo* inhibitor of cell-cycle progression in cultured mammalian cells and that the mitotic arrest in ALLN-treated cells is correlated with inhibition of cyclin B degradation and sustained activation of H1 kinase activity.

The mitotic phenotype of ALLN-treated CHO cells is strikingly similar to that of HeLa cells expressing a nondegradable form of cyclin B (5). Thus, ALLN-treated CHO cells undergo apparently normal processes of chromosome condensation, nuclear-envelope breakdown, development of a mitotic spindle, and congression of chromosomes to the metaphase plate. These cells arrest only at the point that cells would normally undergo anaphase events. The spindle persists for several hours, and chromosome condensation continues during the arrested metaphase. Subsequently, the spindle becomes highly disorganized and multipolar. During the mitotic arrest, cyclin B accumulates to supranormal levels, and histone H1 kinase activity remains elevated relative to controls. At ALLN concentrations permitting eventual progression through mitosis, cyclin degradation and decline in histone kinase activity occur after a delay that is related to ALLN concentration. Nuclei reform in daughter cells containing chromatin, and the chromatin decondenses, although at the light-microscope level, decondensation is abnormal.

Although the inhibition of mitosis by ALLN clearly correlates with significant alterations in the rate of cyclin B proteolysis, our results do not indicate whether this effect results from direct inhibition of a cyclin B protease(s) or a more indirect process. Because cyclin degradation *in vitro* may involve ubiquitination (2–4), our results suggest that a neutral cysteine protease activity may exist in the ubiquitin pathway itself or possibly an activity by which cyclin B is processed before ubiquitination (4). Indirect drug effects are, however, a distinct possibility. In budding yeast signaling pathways exist by which cyclin degradation and maturation-promoting factor activity are modulated in response to spindle integrity/function (14, 15). Such pathways are also pre-
sent in mammalian cells (11). Additionally, intracellular calcium changes occur during mitosis (16), and ALLN inhibits calpains I and II, calcium-dependent neutral cysteine proteinases implicated in mitotic function by experiments in which microinjected calpain II promoted the onset of anaphase in mammalian cells (17). However, in vitro studies of protein degradation in Xenopus oocytes show that calpastatin, a specific inhibitor of calpain, does not inhibit cyclin degradation (18). Thus, the mechanism by which ALLN inhibits cyclin B proteolysis remains to be elucidated.

We also observed that ALLN affects early S-phase progression, and at sufficiently high concentrations ALLN inhibits progression into S-phase altogether. S-phase effects of ALLN are detectable at lower concentrations than mitotic effects. Although we have not yet characterized this effect of ALLN, it is tempting to speculate that ALLN might alter the degradation rate of one or more “G1 cyclins” (19, 20) and thus prolong or block the progression of cells into S phase. Our studies show that ALLN is a potent inhibitor of cell-cycle progression, perturbing the cell cycle at important transition points. The mitotic effect of ALLN is associated directly or indirectly with inhibition of cyclin B degradation and the consequent inactivation of histone H1 kinase activity. Whether ALLN-induced inhibition of cyclin B degradation is direct or indirect, we believe that ALLN provides a useful probe of the mechanisms of cyclin degradation. ALLN may also represent the prototype of a class of antiproliferative agents that operate by specifically inhibiting important cell-cycle regulatory processes, resulting in cell death.

We gratefully acknowledge the generous gift of anticyclin B1 monoclonal antibody from Drs. Ed Harlow and Steven Schiff, Massachusetts General Hospital, cyclin B antiserum from Dr. Tony Hunter, The Salk Institute for Biological Studies, and of anti-calpain II antiserum from Dr. Y. Adachi, Kyoto University, Japan. This work was supported by Grants National Institutes of Health CA 16318 to R.T.S. and HL 26502 to R.D.S.