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Inhibition of Growth of Human TE2 and C-33A Cells by the Cell-Permeant Calpain Inhibitor Benzyloxycarbonyl–Leu–Leu–Tyr Diazomethyl Ketone

RONALD L. MELLGREN,[1] ELLIOTT SHAW,* AND MAURA T. MERICLE

*Department of Pharmacology and Therapeutics, Medical College of Ohio, P.O. Box 10008, Toledo, Ohio 43699-0008; and *Friedrich Miescher Institut, Postfach 2543, CH-4002, Basel, Switzerland*

Calpains are Ca^{2+}-requiring, nonlysosomal proteases which are thought to participate in some aspects of intracellular Ca^{2+}-signal transduction. However, their exact physiologic function has not yet been established. Addition of the cell-permeant, irreversible calpain inhibitor, ZLLY–CHN_2, to human TE2 or C-33A cells inhibited growth, as assessed either by mitochondrial MTT reductase activity or by direct cell counting. Inhibition of growth produced by a 24-h exposure to 50 μM ZLLY–CHN_2 was reversed upon substituting growth medium without inhibitor. Homogenates produced from cells cultured in the presence of ZLLY–CHN_2 displayed decreased calpain and Ca^{2+}-independent proteolytic activities. Protein immunoblot analysis showed that cell cultures which had lost 80% of their calpain activity still retained full calpain immunoreactivity. Therefore, inhibition by ZLLY–CHN_2 appeared to result in accumulation of irreversibly inactivated calpain within the cells. Homogenates from cells cultured in the presence of 20 or 50 μM ZLVG–CHN_2, a cell-permeant inhibitor with little activity against calpains, had decreased Ca^{2+}-independent proteolytic activity, but demonstrated no decrease in calpain activity. ZLVG–CHN_2 did not inhibit cell growth under these conditions. Growth of *Saccharomyces cerevisiae* cells, which do not appear to express calpain-like proteases, was not inhibited by including 50 μM ZLLY–CHN_2 in the culture medium. These results indicate that calpains participate in the social regulation of cell growth in multicellular organisms.

INTRODUCTION

Several recent observations indicate that regulated intracellular proteolysis is an important mechanism for controlling cell growth. The best characterized example for this type of control is the rapid proteolysis of cyclin B in *Xenopus laevis* oocytes following mitotic metaphase. The degradation of cyclin is required for exit from mitosis [1], and appears to be catalyzed by the ubiquitin and ATP-dependent proteolytic pathway [2]. Another cell cycle regulatory protein, p53, appears to be targeted for proteolysis by ubiquitination in human cervical cells transformed with oncogenic papilloma viruses [3].

Another group of regulated intracellular proteases which might be involved in growth control is the calpains, Ca^{2+}-requiring nonlysosomal proteases which are universally distributed in multicellular animals [4–7]. The *m*-calpain isozyme requires millimolar Ca^{2+} for activity *in vitro*, while μ-calpain requires 10^{-5} to 10^{-4} *M* Ca^{2+} [8]. There is considerable evidence that these enzymes can become activated at the substantially lower Ca^{2+} concentrations present inside cells [7, 9, 10]. Recently, calpain activation has been detected within isolated hepatocytes [11] and platelets [12, 13] following stimulation with agents that are known to mobilize intracellular Ca^{2+} stores. In addition to μ-calpain and *m*-calpain, the existence of tissue-specific calpains has also been reported [14, 15].

Since μ- and *m*-calpain are composed of cysteine protease domains and calmodulin-type Ca^{2+} binding domains, there has been considerable interest in their potential function as targets for signal transduction through alteration of Ca^{2+} concentrations. Given the recognized roles of Ca^{2+} signaling and of regulated proteolysis in cell proliferation [16], it seems reasonable that calpains might be involved in cell cycle control, and there is some evidence for their participation. Microinjection of *m*-calpain into cultured PtK_1 cells was reported to accelerate mitosis [17]. In addition, HTLV-I infection of T-lymphocytes and some B-lymphocytes induced expression of *m*-calpain in these cells [18], and it was suggested that this may be related to the Ca^{2+}-dependent growth characteristics of HTLV-I-infected cells.

A major obstacle in assessing the role of calpains in cell growth is the lack of specific, cell-permeant calpain antagonists. The tripeptide aldehyde acetyl–Leu–Leu–Norleucinal (CI-1), a potent inhibitor of calpains, has recently been shown to block the serum-stimulated growth of synchronized smooth muscle cells at the G_1/S and G_2/M boundaries [19]. However, CI-I inhibits many

[1] To whom reprint requests should be addressed.

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cysteine proteases and some other proteases, including the proteasome, an essential component of ubiquitin-dependent proteolysis [20]. Therefore, its anti-proliferative action cannot be unambiguously ascribed to inhibition of calpains. E64-d, a cell-permeant irreversible inhibitor of thiol proteases, produced a mitotic arrest of A431 human epidermoid carcinoma cells [21]. Unfortunately, it was not possible to identify a specific target associated with its anti-proliferative effect, since a number of cell proteins were labeled by radioactive E64-d [21].

Diazomethyl ketones are selective inhibitors of cysteine proteases [22]. A tripeptidyl diazomethyl ketone, benzyloxycarbonyl–Leu–Leu–Tyr–diazomethyl ketone (ZLLY–CHN$_2$), was shown to be a rapid, irreversible inhibitor of calpains and cathepsin L [23]. Cathepsin B was much less susceptible to inhibition. Recently, it was demonstrated that radioactive ZLLY–CHN$_2$ labeled calpain large subunit in intact platelets upon addition of a Ca^{2+} ionophore [24, 25]. Cathepsin L forms were the only other major labeled protein bands. Relative to previously available inhibitors, this represents a substantial improvement in specificity toward calpains. Other cell-permeant diazomethyl ketones are potent inhibitors of cathepsin L but do not effectively inhibit calpains [23]. Thus, by the use of appropriate controls, it should be possible to dissect effects related to inhibition of calpains from those due to inhibition of cathepsin L. In the present study, we have utilized these novel reagents to investigate the potential role of calpains as regulators of cell growth in cultured human cells.

MATERIALS AND METHODS

Materials. Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco, and defined calf serum was obtained from Hyclone. Cell culture grade dimethyl sulfoxide (DMSO) (catalog D5879), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), benzyloxycarbonyl–Leu–Val–Gly diazomethyl ketone (ZLVG–CHN$_2$), phenylmethylsulfonyl fluoride (PMSF), TPCK-treated trypsin, Hammersten casein, and soybean trypsin inhibitor were obtained from Sigma. Benzyloxycarbonyl–Leu–Leu–Tyr diazomethyl ketone (ZLLY–CHN$_2$) and benzyloxycarbonyl–Phe–Ala diazomethyl ketone (ZFA–CHN$_2$) were synthesized from the appropriate acid anhydride and ethereal diazomethane [23]. Human erythrocyte μ-calpain [26], human erythrocyte multicatalytic protease [27], and monoclonal antibodies against calpains and calpastatin [28, 29], were prepared as previously described. Casein to be used as a protease substrate was labeled with [^{14}C]formaldehyde by reductive methylation [30].

Mammalian cell culture. TE2 cells, SV40-transformed human esophageal epithelial cells, were a kind gift from Dr. Gary Stoner (Department of Preventive Medicine, Ohio State University). C-33A cells, derived from a human cervical carcinoma, were obtained from the American Type Culture Collection (Rockville, MD). All cells were cultured at 37°C in a 5% CO_2 atmosphere in IMDM supplemented with L-glutamine, 25 m*M* Hepes, and 10% calf serum.

Yeast culture. Strain Y466 *Saccharomyces cerevisiae* was kindly supplied by Dr. Robert Trumbly, Department of Biochemistry. Yeast were grown in a rich, uracil-containing medium at 30°C. Additions of vehicle or protease inhibitor were made when a cell density corresponding to 0.1 A_{600} unit was obtained.

Cell growth assay. Cell growth was estimated by measurement of mitochondrial MTT reductase activity as previously described [31], with minor modifications. Cells were grown in 24-well plates in 1 ml of medium. MTT was added to a final concentration of 0.83 mg/ml, and the cells were incubated for 30 min at 37°C. Culture medium was removed and placed in a 1.5-ml Eppendorf microcentrifuge tube. After centrifugation at 10,000*g* for 2 min, the supernatants were removed. Cells which remained attached to the culture well were suspended in 800 μl of 95% ethanol to solubilize the blue formazan product of MTT reduction. The ethanol suspension was then transferred to the tubes containing the pellets recovered from the culture medium, and mixed by vortexing. This effectively removed the blue dye from the pellets. The samples were centrifuged once more and the A_{600} of the final supernatant was recorded.

Cell proliferation. Proliferative cell growth was measured directly by counting suspended trypsinized cells using a hemocytometer.

Effect of protease inhibitors on cell growth. Cells were seeded in 24-well culture plates at a density of approximately 6×10^4 cells/well in 200 μl of medium and incubated overnight to allow attachment. Cells were then cultured in a total of 1 ml of medium containing protease inhibitor dissolved in DMSO, or an equivalent volume of DMSO alone for controls. Usually, the final DMSO concentration was 0.1%, and it was never greater than 0.25%. At 24-h intervals triplicate wells were examined for cell growth, as described above. Every 24 h, 0.5 ml of culture medium was removed, and replaced with fresh medium containing vehicle, or protease inhibitor at the appropriate concentration.

Preparation of cell homogenates. Cells were grown in 5 ml of IMDM in 25-cm^2 flasks until within approximately 50% of confluence, and then treated with vehicle or protease inhibitor. Incubations were terminated by washing the cells two times with 5 ml of Hank's balanced salt solution at 37°C and scraping the cells in 0.5 ml of ice-cold 50 m*M* Mops, 5 m*M* EDTA, 0.5% Triton X-100, pH 6.5. The cells were then homogenized in a small frosted-glass homogenizer. To one-half of the homogenate, dithiothreitol was immediately added to 1 m*M*, to stabilize calpains and other thiol proteases prior to assay (see below). The sample without dithiothreitol was used for protein determinations.

Protein immunoblotting. Proteins and cell homogenates were heated to 100°C and subjected to SDS–PAGE on 10% slab gels using the Laemmli buffer system [32]. Proteins in the gels were transferred to nitrocellulose sheets and immunostained for calpain or calpastatin as previously described using alkaline phosphatase-conjugated second antibody, and indolyl phosphate/nitroblue tetrazolium for detection [33]. μ-Calpain large subunit was detected with 7H-4 monoclonal antibody, which does not cross-react with *m*-calpain large subunit. Calpain small subunit, which is the same gene product whether present in *m*-calpain or μ-calpain, was detected with P-1 monoclonal antibody. Calpastatin was detected with 5-8A monoclonal antibody, which was obtained using human erythrocyte calpastatin as the antigen.

Protein determination. The protein concentration of cell homogenates was determined by the BCA method [34] using bovine serum albumin as the standard.

Calpain assay. Calpain activity was determined by release of trichloroacetic acid-soluble fragments from [^{14}C]methyl casein essentially as previously described [35]. A unit of calpain solubilizes 1 μg of casein per minute. For determination of activity in cell homogenates, 10 μl of homogenate was assayed for 20 min in the presence of 5 m*M* Ca^{2+}. To correct for background proteolysis in cell homogenates, blanks contained all of the components of the assay mixture including the cell homogenates, as well as 16 μg human calpastatin/ml. The latter is a highly specific inhibitor of calpains [36]. Under the condi-

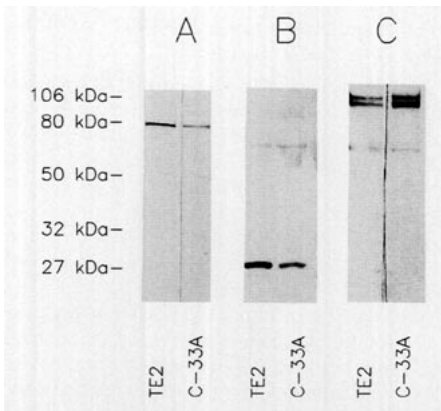

FIG. 1. Calpain and calpastatin content of TE2 and C-33A cells. Cell homogenates were subjected to SDS–PAGE and protein immunoblotting for μ-calpain large subunit (A), μ- and m-calpain small subunits (B), or calpastatin (C), as described under Materials and Methods. Each lane contains 20 μg of total protein.

tions of calpain assay, background proteolysis accounted for no more than 30% of the total caseinolytic activity.

Multicatalytic protease complex caseinolytic activity. Purified multicatalytic protease complex was activated by preincubation with polylysine and assayed as previously described using [^{14}C]casein as the substrate [27].

Calcium-independent caseinolytic activity. Ten-microliter aliquots of cell homogenate were incubated in a final volume of 50 μl with 50 mM Mops, 1 mM EDTA, 1 mM dithiothreitol, 0.4 mg [^{14}C]casein/ml, pH 6.2. After incubation at 37°C for 120 min, reaction was terminated by addition of trichloroacetic acid to 2.5%. Samples were centrifuged at 12,000g for 5 min, and one-half of the supernatant was spotted on Whatman 31ET filter paper, dried under a heat lamp, and counted in a liquid scintillation counter. One unit of Ca^{2+}-independent protease solubilizes 1 μg of casein per minute.

Calpastatin assay. Samples of cell homogenates were heated to 95°C for 10 min to inactivate endogenous calpains, and centrifuged at 10,000g for 10 min. The resulting supernatant was assayed for calpastatin by its ability to inhibit the caseinolytic activity of purified bovine myocardial m-calpain, as previously described [37].

RESULTS

Characterization of the calcium-dependent proteolytic system in TE2 and C-33A cells. Protein immunoblot analysis of cell homogenates showed that both cell lines used in this study expressed calpains and calpastatin (Fig. 1). However, TE2 cells clearly contained more μ-calpain (Fig. 1A), more total calpain (Fig. 1B), and less calpastatin (Fig. 1C). Calpastatin appeared as a doublet of immunoreactivity in either cell line. This is apparently the result of alternative splicing of calpastatin mRNA [38].

ZLLY–CHN$_2$ inhibits the growth of TE2 and C-33A cells. Addition of ZLLY–CHN$_2$ to the culture medium resulted in a dose-dependent inhibition of the growth of TE2 cells (Fig. 2A) and C-33A cells (Fig. 2B) determined by MTT reductase activity as described under Materials and Methods. Treatment of TE2 cells with 50 μM ZLLY–CHN$_2$ for periods longer than 48 h frequently produced toxicity (Fig. 2A). C-33A cells were more stable under these conditions.

Direct cell counting confirmed that cell proliferation was totally inhibited by 50 μM ZLLY–CHN$_2$ (Fig. 3). Treatment of TE2 cells and C-33A cells with this inhibitor for 48 h appeared to produce a small loss of cells compared to the initial number present (Fig. 3). Although this effect is not significant in the data presented in Fig. 3, a number of experiments have shown that some cells become detached during treatment with ZLLY–CHN$_2$ for periods longer than 24 h. Only in rare instances did more than 30% of the total cell number become detached. Upon replating in the absence of inhibitor, approximately one-half of the cells subsequently attached and eventually continued growth. Therefore, detachment does not necessarily represent an irreversible toxicity. All further experiments described deal only with the cells which remained attached to culture plates after treatment.

Inhibition of growth by a 24-h exposure to 50 μM

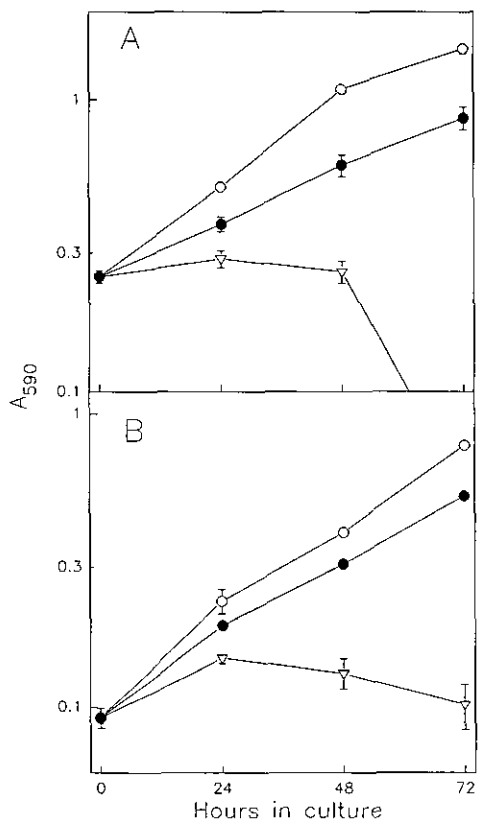

FIG. 2. Inhibition of cell growth by ZLLY–CHN$_2$. TE2 cells (A) or C-33A cells (B) were cultured in the presence of ZLLY–CHN$_2$. At indicated periods of time, cell growth was assessed as described under Materials and Methods. Open circles, no inhibitor; filled circles, 20 μM ZLLY–CHN$_2$; triangles, 50 μM ZLLY–CHN$_2$. Data represent means and standard deviation of three wells for each experimental condition.

ZLLY-CHN_2 could be fully reversed by allowing the cells to recover in culture medium without inhibitor (Fig. 4). The same result was obtained by direct cell counting, although recovery was not as rapid (data not shown).

Changes in protease activities after exposure of cells to ZLLY-CHN_2. Because peptidyl diazomethyl ketones are irreversible inhibitors of thiol proteases [22], it was possible to determine the degree of inactivation of calpains and Ca^{2+}-independent proteases produced by exposing cells to this inhibitor. The high content of calpastatin in C-33A cells (Fig. 1) precluded an accurate measurement of calpain activity in cell homogenates. Nevertheless, some assays were carried out after incubation of C-33A cell homogenates with 50 μg trypsin/ml to deplete endogenous calpastatin, followed by addition of excess trypsin inhibitor. These studies indicated that at least one-half of the calpain activity was inactivated after a 48-h exposure of C-33A cells to 50 μM ZLLY-CHN_2.

TE2 cells contained more calpain than calpastatin, and activity could be readily determined by direct assay of cell homogenates. TE2 cells were cultured in the presence of 20 or 50 μM ZLLY-CHN_2 for 18 h, and homogenates were prepared and assayed for proteolytic activities as described under Materials and Methods. Total calpain activity was extensively decreased upon exposure of TE2 cells to ZLLY-CHN_2 (Fig. 5). Approximately one-half of the total calpain activity was depleted by incubation of TE2 cells with 20 μM ZLLY-

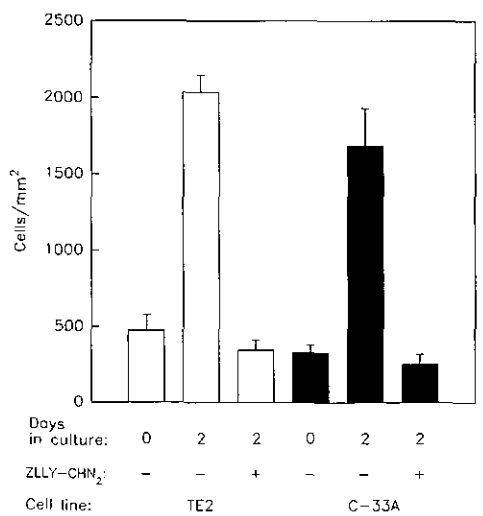

FIG. 3. Inhibition of cell proliferation by ZLLY-CHN_2. TE2 cells (open bars) or C-33A cells (solid bars) were cultured for 2 days in the absence or presence of 50 μM ZLLY-CHN_2, and removed from the culture wells by trypsin treatment. The cell density was determined using hemocytometer. Initial cell counts were taken immediately prior to adding ZLLY-CHN_2. Data represent means and standard deviation of at least five separate counts from each of three culture wells.

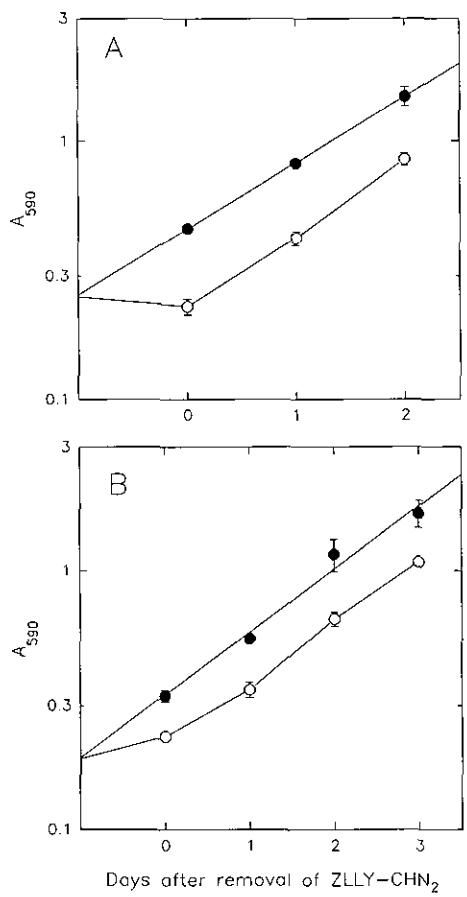

FIG. 4. Reversibility of cell growth inhibition. TE2 cells (A) or C-33A cells (B) were cultured with the addition of DMSO alone (solid circles) or 50 μM ZLLY-CHN_2 (open circles) for 24 h. The medium was then replaced with IMDM containing no inhibitor, and growth was monitored for an additional 2 days (A) or 3 days (B). Means plus standard deviation are presented (N = 6 for controls, N = 3 for treated samples).

CHN_2. Protein immunoblots showed that there was no change in the content of μ-calpain large subunit or calpain small subunit in the samples (Fig. 6). Since both calpain isozymes contain the same small subunit [39], the latter experiment indicated that there was no depletion of total calpain protein in the inhibitor-treated samples. Similarly, calpastatin assays performed as described under Materials and Methods indicated that all samples had approximately the same low level of calpastatin activity (not shown). It is most probable that the loss of calpain activity was the result of its covalent modification by reaction with the diazomethane group in ZLLY-CHN_2 [22].

Calcium-independent caseinolytic activity, assayed as described under Materials and Methods, was inactivated only to a modest extent in cells treated with ZLLY-CHN_2 (Fig. 7). Maximum inhibition occurred at 20 μM ZLLY-CHN_2 or less with either cell line. Further

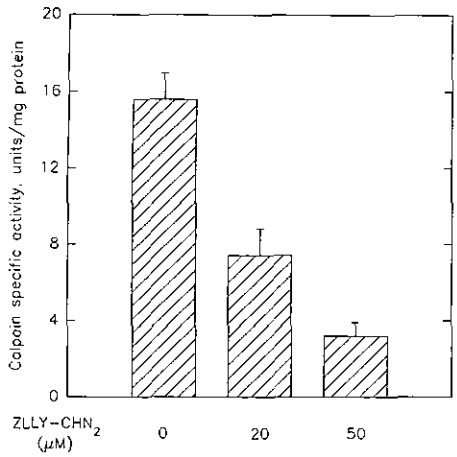

FIG. 5. Inactivation of calpain following exposure of TE2 cells to ZLLY–CHN_2. TE2 cells were cultured for 18 h in 25-cm^2 culture flasks in the presence of various concentrations of ZLLY–CHN_2. The cells were extensively washed with Hank's balanced salt solution, and cell homogenates were prepared and assayed for proteolytic activities as described under Materials and Methods. Data represent the mean and standard deviation of three flasks of cells.

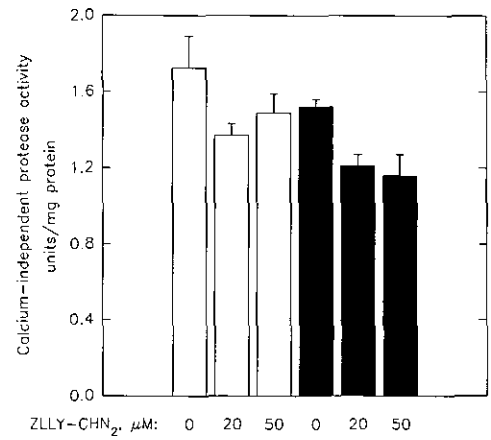

FIG. 7. Inactivation of Ca^{2+}-independent caseinolytic activity following exposure of cells to ZLLY–CHN_2. TE2 cells (open bars) and C-33A cells (solid bars) were cultured in the presence of ZLLY–CHN_2 as described in Fig. 5. Samples were assayed for Ca^{2+}-independent caseinolytic activity as described under Materials and Methods.

studies indicated that most of the caseinolytic activity in C-33A cells (Table 1) and TE2 cells (not shown) was inhibitable by pepstatin A, and was therefore probably due to an aspartyl protease, which would not be sensitive to cysteine protease inhibitors like ZLLY–CHN_2. As expected, treatment of cells with 50 μM ZLLY–CHN_2 inactivated the minor fraction of caseinolytic activity in cell homogenates which was sensitive to this inhibitor, while having no effect on pepstatin A-sensitive activity (Table 1).

Effect of other cell-permeant cysteine protease inhibitors on cell growth. While ZLLY–CHN_2 is a very effective inhibitor of purified *m*-calpain [23] and μ-calpain (Fig. 8), another peptidyl diazomethyl ketone, ZLVG–CHN_2, is 30-fold less potent under standard assay conditions (Fig. 8). ZLVG–CHN_2 appears to inactivate an intracellular thiol protease in intact group A *Streptococci* cells [40], and is a potent inhibitor of cysteine cathepsins, including cathepsin L [41]. Thus, it should be capable of inhibiting cathepsin L in intact cultured cells. As anticipated, addition of ZLVG–CHN_2 to TE2 cells in culture resulted in a modest degree of inactivation of Ca^{2+}-independent caseinolytic activity, but had no effect on calpain activity (Fig. 9). Addition of 50 μM ZLVG–CHN_2 to the culture medium of TE2 or C-33A cells produced virtually no effect on cell growth assessed by MTT reductase activity (Fig. 10). In another experiment, TE2 cells were cultured in the presence of 50 μM concentrations of ZLVG–CHN_2 or ZFA–CHN_2, another potent inhibitor of cathepsin L which has little calpain inhibitory activity [23]. Neither inhibitor significantly altered cell proliferation (Fig. 11).

Effect of ZLLY–CHN_2 on the growth of yeast cells. Many of the enzymes which regulate eukaryotic cell pro-

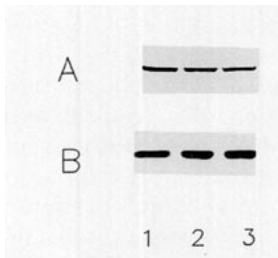

FIG. 6. Calpain large and small subunit immunoreactivity in TE2 cells cultured in the presence of ZLLY–CHN_2. The cell homogenate samples described in Fig. 5 were subjected to protein immunoblotting using monoclonal antibodies against μ-calpain large subunit (A) or the small subunit present in both μ- and *m*-calpains (B). The blot used in this figure is representative of the two other samples. Lane 1, no ZLLY–CHN_2; lane 2, 20 μM ZLLY–CHN_2; lane 3, 50 μM ZLLY–CHN_2.

TABLE 1

Susceptibility of Ca^{2+}-Independent Caseinolytic Activity in C-33A Cell Homogenates to Various Protease Inhibitors

	% Inhibition	
Inhibitor, conc.	Cultured − ZLLY-CHN_2	Cultured + ZLLY-CHN_2
PMSF, 100 μM	<10	<10
Pepstatin A, 10 μM	79	86
ZLLY-CHN_2, 10 μM	20	<10

Note. Cells were cultured in the absence or presence of 50 μM ZLLY-CHN_2 for 48 hours, washed to remove ZLLY-CHN_2 in the culture medium, homogenized, and assayed as described under Materials and Methods for Ca^{2+}-independent proteolytic activity.

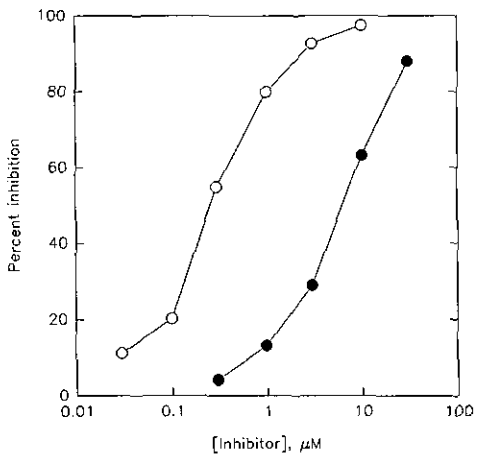

FIG. 8. Inhibition of purified μ-calpain by peptidyldiazomethyl ketone protease inhibitors. Purified μ-calpain at a concentration of 20 n*M* was assayed for caseinolytic activity for 10 min in the presence of various concentrations of ZLLY-CHN_2 (open circles) or ZLVG-CHN_2 (filled circles).

liferation are highly conserved, and found in yeast as well as higher organisms [42]. We therefore investigated possible inhibition of the growth of *S. cerevisiae* by including 50 μ*M* ZLLY-CHN_2 in the culture medium and allowing the organisms to grow for more than two doubling times under conditions described under Materials and Methods. Three cultures grown for 5 h without inhibitor had A_{600} values of 0.483 ± 0.006 SD; while three cultures grown in the presence of 50 μ*M* ZLLY-CHN_2 had A_{600} values of 0.503 ± 0.004 SD. When cultured several days to achieve stationary phase, the control and ZLLY-CHN_2-treated yeast grew to A_{600} values of 10.7 and 9.5, respectively. Therefore, concentrations of ZLLY-CHN_2 which stopped growth of C-33A or TE2 cells did not inhibit growth of *S. cerevisiae*.

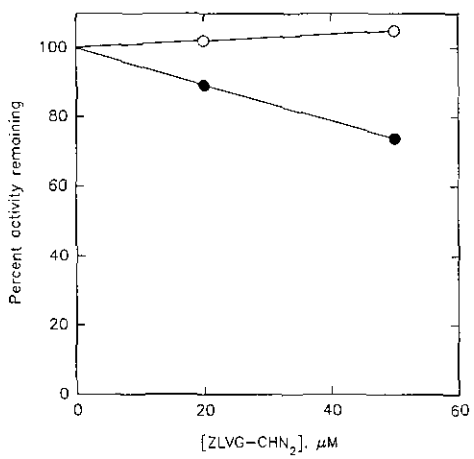

FIG. 9. Inactivation of proteases in TE2 cells cultured in the presence of ZLVG-CHN_2. Cells were cultured in the presence of ZLVG-CHN_2 for 18 h. Cell homogenates were prepared and assayed for calpain (open circles) or Ca^{2+}-independent proteolytic activity (filled circles) as described under *Materials and Methods*. The data are means of values from two culture flasks for each condition. The two assays for each data point differed by less than 10%.

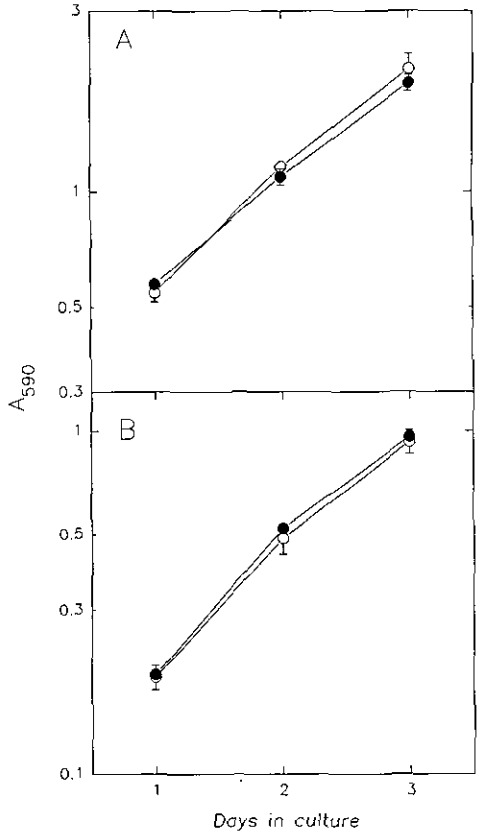

FIG. 10. Failure of ZLVG-CHN_2 to inhibit growth of TE2 or C-33A cells. TE2 cells (A) or C-33A cells (B) were cultured in the presence of DMSO alone (open circles) or 50 μ*M* ZLVG-CHN_2 (filled circles). At the indicated times, samples were analyzed for cell growth. Means ± SD for triplicate wells at each time point.

DISCUSSION

The relative amounts of the two major calpain isozymes and calpastatin vary considerably from one cell type to another [4]. This variability in content may reflect specific functions of calpains in particular cell types, or cell-selective mechanisms for regulation of calpain activity. On the other hand, since some level of calpain expression is evident in all cells, it seems likely that there are functions common to all cells that are supported by calpain. To determine whether regulation of cell growth might be such a common function, we have investigated the influence of the calpain inhibitor ZLLY-CHN_2 on the growth of both TE2 cells and C-33A cells, two human cell lines differing in calpain

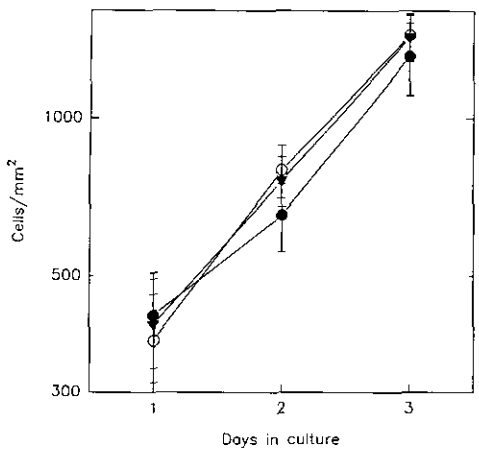

FIG. 11. Failure of ZLVG–CHN_2 or ZFA–CHN_2 to inhibit proliferation of TE2 cells. Cells were grown in the presence of 50 μM ZLVG–CHN_2 (solid circles), 50 μM ZFA–CHN_2 (triangles), or no additions (open circles). Means ± SD of at least five separate cell counts for each of three separate wells.

and calpastatin content (Fig. 1). The results show that growth of both cell types is arrested by including 50 μM ZLLY–CHN_2 in the culture medium.

ZLLY–CHN_2 and other cell permeable peptidyl diazomethyl ketones are irreversible, mechanism-based inhibitors of thiol proteases [44]. Thus, it was possible to assess depletion of protease activities after culturing cells in the presence of inhibitors. The results of these studies suggest that ZLLY–CHN_2 inhibits cell growth by inactivation of endogenous calpain activity. Calpain activity was depleted in cells cultured in the presence of ZLLY–CHN_2 (Fig. 5), while calpain immunoreactivity was not affected (Fig. 6). This is consistent with covalent inactivation of calpains by reaction with the diazomethyl ketone. The cell-permeant diazomethyl ketones, ZLVG–CHN_2 and ZFA–CHN_2, which are excellent inhibitors of thiol cathepsins, but poor calpain inhibitors, were not capable of inhibiting cell growth (Figs. 10 and 11). Therefore, the inhibition of cell growth by ZLLY–CHN_2 did not appear to be a nonspecific affect of peptidyl diazomethyl ketones, or the result of inhibition of other thiol proteases.

Preliminary studies showed that growth of CHO-K1 hamster fibroblasts, mouse L929 fibroblasts, rat PC12 pheochromocytoma-derived cells, and Sf-9 insect ovary cells was arrested when they were cultured in the presence of 50 μM ZLLY–CHN_2. In contrast, growth of the yeast *S. cerevisiae* was unaffected. This is an important observation for at least two reasons. First, neither calpains nor calpain-like genes have been demonstrated in yeast. Experiments in the authors' laboratory applying the standard protocols utilized for calpain purification have failed to recover significant Ca^{2+}-dependent caseinolytic activity from *S. cerevisiae* extracts. Therefore, the observed result is consistent with the hypothesis that ZLLY–CHN_2 produces inhibition of growth by inactivating calpains. Second, yeast contain highly conserved analogs of many of the proteins known to control cell proliferation in animal cells, including cyclins, cell cycle-related protein kinases, and the ubiquitin-dependent proteolytic system [45, 46]. Failure of ZLLY–CHN_2 to inhibit growth of *S. cerevisiae* argues against its inhibition of these cell cycle regulatory proteins, including the proteasome, the proteolytic component of the ubiquitin-dependent proteolytic system [46, 47]. This is consistent with its ability to inhibit purified human erythrocyte proteasome only at very high concentrations (half-maximum inhibition at >50 μM ZLLY–CHN_2, R. Mellgren, unpublished data).

ZLLY–CHN_2 inhibits cathepsin L as well as calpains, and in fact the rate constant for its inactivation of cathepsin L is much higher than for *m*-calpain [23]. However, our experimental observations argue against cathepsin L as a target for its growth inhibitory properties. First, high concentrations of ZLLY–CHN_2 were required to inhibit growth of TE2 or C-33A cells. The requirement of 50 μM ZLLY–CHN_2 for full inhibition of growth is consistent with the concentrations required to inhibit calpain activity in intact platelets [24]. Because of its greater sensitivity to ZLLY–CHN_2 *in vitro*, one would expect an affect on cathepsin L to occur at lower concentrations of inhibitor. Second, and more importantly, cell permeant inhibitors of cathepsin L having little or no activity on calpains were incapable of inhibiting cell growth (Fig. 10) or cell proliferation (Fig. 11).

While one cannot totally exclude the possibility that ZLLY–CHN_2 inhibits some as yet unidentified regulator of cell proliferation, suppression of cell growth by an established, mechanism-directed inhibitor of calpains argues for the participation of *m*-calpain, μ-calpain, or some other member of the calpain gene family [14, 15], in growth regulation in TE2 and C-33A cells. Further studies to identify the specific calpain isozyme involved are underway. Given that the calpains are Ca^{2+}-responsive enzymes which appear to be present only in multicellular animals, it is conceivable that they function in the social regulation of cell growth that is necessary for maintaining organization and viability in complex organisms.

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