

# The Major Calpain Isozymes Are Long-lived Proteins

DESIGN OF AN ANTISENSE STRATEGY FOR CALPAIN DEPLETION IN CULTURED CELLS\*

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**Calpains are intracellular  $\text{Ca}^{2+}$ -dependent proteases that are thought to participate in  $\text{Ca}^{2+}$ -associated signal transduction pathways. It has been proposed that calpains are activated by an autoproteolytic mechanism. If this is true one would expect a relatively short half-life for calpain protein in cells. To test this hypothesis, WI-38 human diploid fibroblasts were pulse-labeled with [ $^{35}\text{S}$ ]methionine, and calpain was immunoprecipitated at various times after chasing with nonradioactive methionine to determine residual radioactivity. The results demonstrated that the two major calpain isozymes, m-calpain and  $\mu$ -calpain, had metabolic half-lives of approximately 5 days. Calpains were long-lived proteins in several human cell lines, A-431, HeLa, VA-13, C-33A, and TE2 cells. In addition, calpastatin, the calpain-specific inhibitor protein, also had a long metabolic half-life. These observations suggest that the model for calpain activation by autoproteolysis requires re-investigation. Based on a knowledge of calpain metabolic stability, a protocol was devised for chronic exposure of WI-38 cells and HeLa cells to a calpain small subunit antisense oligodeoxyribonucleotide. Depletion of calpain small subunit after 5 or more days of treatment led to inhibition of cell proliferation that could be reversed by removal of antisense oligodeoxyribonucleotide from the culture medium. Together with previous studies, these results indicate a requirement for calpains in mammalian cell proliferation.**

Calpains are  $\text{Ca}^{2+}$ -requiring intracellular cysteine proteases that are widely, perhaps universally, distributed in cells of multicellular animals (1–5). All mammalian cells studied thus far appear to contain one or both of the two major calpain isozymes, m-calpain, which requires millimolar  $\text{Ca}^{2+}$  for activity in extracts, and  $\mu$ -calpain, which requires 10–100  $\mu\text{M}$   $\text{Ca}^{2+}$  (5, 6). There are also tissue-specific forms of calpain (7), and most cells contain an inhibitor protein, calpastatin (8), which is highly specific for the calpains (9). There is considerable variability in the calpain isozyme and calpastatin contents of different cell types (4).

While their exact physiologic function has not been established, it is likely that calpains are required for some  $\text{Ca}^{2+}$ -de-

pendent signal transduction pathways. They possess calmodulin-like  $\text{Ca}^{2+}$  binding domains, and calmodulin binding proteins are excellent calpain substrates (10, 11). The nature of proteolysis makes it likely that calpains are involved in regulating events that are irreversible, at least within the time frame of *de novo* protein synthesis. It is easy to imagine that calpains might extend calmodulin-like signaling to include long term events. There has been interest in the potential involvement of calpains in differentiation (12–15) and cell cycle regulation (16–18).

Several years ago, it was proposed that calpains are proenzymes that become activated through an autoproteolytic mechanism (1, 19). While this hypothesis has not received universal support (20, 21), it has formed the basis for attempts to assess calpain activation by appearance of autoproteolyzed calpain forms (22, 23). Autolytic fragments of calpains are not detectable in healthy cells when precautions are taken to avoid post-homogenization proteolysis (22, 24). This implies that, if formed, the autolysis products are rapidly degraded by downstream proteolytic mechanisms. Thus, if calpains are reasonably active within cells, the autolysis model suggests that they should have relatively short metabolic half-lives. To address this issue, and as a preliminary study to establish conditions for calpain depletion by AS-ODN<sup>1</sup> methodology, we have investigated the metabolic stability of calpains in WI-38 fibroblasts and several human cell lines. In addition to showing that calpains are relatively stable proteins, these studies have allowed the development of a protocol for depletion of calpain in cultured cells by chronic exposure to a calpain AS-ODN.

## EXPERIMENTAL PROCEDURES

**Materials**—Electrophoresis supplies and nitrocellulose used in immunoblotting were obtained from Bio-Rad. Trans<sup>35</sup>S-Label<sup>TM</sup>, metabolic labeling reagent containing 70% of the label as [ $^{35}\text{S}$ ]Met, >1000 Ci/mmol, was obtained from ICN. IMDM, DMEM, Opti-MEM I, and Met-free DMEM were purchased from Life Technologies, Inc. Bovine serum and fetal bovine serum were from HyClone. Alkaline phosphatase-conjugated anti-mouse IgG, 3-indolyl phosphate, nitroblue tetrazolium, and MTT were purchased from Sigma. Protein A/G agarose was obtained from Pierce. Phosphorothioate AS-ODNs were synthesized by Oligos Etc. Calpain AS-ODN (AGGAACATGGCTGCGACTCAC) and calpastatin AS-ODN (TGTGGGATTCATACTGGAAGA) were antisense to the initiation Met regions of human calpain small subunit and human calpastatin mRNAs, respectively. A “random” phosphorothioate ODN (CGGCACGCAACCTAGAAGTGT) containing the same base composition as calpain AS-ODN was also synthesized. These ODN sequences and their complementary sequences were not found in any

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EBI Data Bank with accession number(s) X04366 and X04106.

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<sup>1</sup> The abbreviations used are: calpain AS-ODN, phosphorothioate oligodeoxyribonucleotide which is antisense to human calpain small subunit mRNA; ODN, oligodeoxyribonucleotide; IMDM, Iscove’s modified Dulbecco’s medium; DMEM, Dulbecco’s modified Eagle medium; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ZLLY-CHN<sub>2</sub>, benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone; Mops, 4-morpholineethanesulfonic acid.

nucleic acid, other than the targets of the AS-ODNs, in a search utilizing the BLASTN program from the National Center for Biotechnology Information. ZLLY-CHN<sub>2</sub> was kindly provided by Dr. Elliott Shaw, Friedrich Miescher Institut, Basel.

**Antibodies**—The following antibodies were employed in the immunoprecipitation studies described in this work. P-1 is a mouse monoclonal antibody that recognizes calpain small subunit in either major isozyme (25). A mouse monoclonal antibody developed against human  $\mu$ -calpain large subunit, P-9, did not cross-react with human placental m-calpain. m-Calpain large subunit was immunoprecipitated with PC-1 rabbit immune serum that did not recognize  $\mu$ -calpain large subunit in immunoblots (26). Preliminary studies showed that it did not efficiently immunoprecipitate purified human erythrocyte  $\mu$ -calpain under the conditions used in our studies, but it recognized calpain small subunit in protein immunoblots. Calpastatin was immunoprecipitated with PI-8 mouse monoclonal antibody.

**Cell Culture**—WI-38 human diploid fibroblasts, HeLa cells, and VA-13, and SV40-transformed WI-38 cells were routinely cultured in DMEM containing 10% fetal bovine serum. A-431, C-33A, and TE2 cells were cultured in IMDM containing 10% bovine serum.

**Metabolic Labeling and Immunoprecipitation of Calpain Subunits**—Cells were grown in monolayer cultures, usually until 60–70% confluent, in 35-mm plastic culture dishes. They were washed several times with phosphate-buffered saline, and then methionine-free DMEM containing 10% dialyzed fetal bovine serum was added. After 1 h, 100  $\mu$ Ci [<sup>35</sup>S]Met was added. Cells were labeled for 12 h and then placed in the usual culture medium, which contained 0.2 mM nonradioactive Met. At zero time, and various times after removal of the [<sup>35</sup>S]Met, the cells were lysed in 0.2 ml of lysis buffer (50 mM Tris-HCl, 0.5 M NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 2 mM EGTA, 20  $\mu$ M leupeptin, and 10  $\mu$ M phenylmethylsulfonyl fluoride, pH 7.5). The cell lysate was incubated on ice for 30 min and centrifuged at 10,000  $\times g$  for 20 min at 4 °C. To the resultant supernatant, SDS was added to 0.5%, and the sample was heated to 95 °C for 3 min to denature the proteins. The heated supernatant was diluted with four volumes of lysis buffer without Nonidet P-40, but containing 0.625% deoxycholate, and pretreated with 20  $\mu$ l of protein A/G-agarose bound with normal rabbit IgG prior to immunoprecipitation.

For immunoprecipitation of calpains, the pretreated samples were incubated for 20 h with 20  $\mu$ l of protein A/G-agarose containing bound antibodies against calpain small subunit,  $\mu$ -calpain large subunit, or m-calpain large subunit. Preliminary experiments indicated that under these conditions calpain antibodies completely precipitated the relevant antigen from the cell extracts. The gels were washed four times with lysis buffer without Nonidet P-40 and twice with phosphate-buffered saline. Three-fold diluted SDS-sample buffer was added, and the samples were heated at 95 °C for 5 min and centrifuged to separate eluted proteins from the gel. Aliquots were applied to 10% polyacrylamide slab gels, electrophoresed, and subjected to autoradiography to visualize <sup>35</sup>S-labeled protein bands. A standard curve for estimating relative amounts of radiolabeled calpain in autoradiograms was prepared as follows. Different amounts of <sup>35</sup>S-labeled proteins present in WI-38 cell lysates were loaded on a gel. The densitometric readings of several individual radioactive bands were graphed *versus* amount of cell lysate loaded. All of the curves displayed similar shapes, and a composite curve was prepared from their averaged values. This standard curve was used to estimate the percent loss of calpain and calpastatin <sup>35</sup>S label with time.

In a control experiment, the Met concentration in the chase medium was increased 10-fold. The higher concentration had no effect on the measured loss of [<sup>35</sup>S]Met from  $\mu$ - or m-calpain large subunit or calpain small subunit. Therefore, the half-life values do not appear to be affected by re-utilization of isotope.

**Metabolic Labeling and Immunoprecipitation of Calpastatin**—Cells were labeled and lysed as described above for calpains. Cell lysate was then heated to 95 °C for 5 min without addition of SDS. Denatured proteins were removed by centrifugation and discarded. Calpastatin is not denatured by this treatment and remains in the supernatant fraction, which was then subjected to immunoprecipitation, electrophoresis, and autoradiography as described in the preceding section, except that a calpastatin antibody was used.

**Antisense Treatment of Cells**—WI-38 fibroblasts or HeLa cells, at approximately 20–30% confluence, were cultured in the presence of 0.2  $\mu$ M phosphorothioate ODNs as a complex with 5  $\mu$ g of LipofectAMINE/ml. The ODN-LipofectAMINE complex was prepared in Opti-MEM I buffer in the absence of serum as described in the LipofectAMINE product insert. After 30–45 min in Opti-MEM, to allow complex formation, 4 volumes of DMEM containing 10% heat-inactivated (56 °C, 1 h)

fetal bovine serum was added. Cells were cultured in 0.5 of the ODN-containing medium, and the remainder was kept refrigerated for use the following day; medium was changed every day and fresh medium was prepared every 2nd day.

**Immunoblotting**—Cells in 6-well culture plates were washed with four successive 2.5-ml samples of Hanks' buffered saline solution warmed to 37 °C. The culture plates were then placed on ice, and the cells were suspended by scraping in 0.25 ml of 20 mM Mops, 150 mM NaCl, 5 mM EDTA, 10  $\mu$ M pepstatin A, 0.25 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, pH 7.0. They were further homogenized 10 strokes in a small frosted glass homogenizer and centrifuged for 10 min at 5000  $\times g$  in a refrigerated Sorvall centrifuge. Samples of the supernatants were heated to 100 °C for 5 min in SDS-sample buffer and electrophoresed in a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose and immunostained for calpain small subunit using the P-1 monoclonal antibody. Alkaline phosphatase-conjugated anti-mouse IgG was used as second antibody, and the immunoreactive bands were detected with indolyl phosphate and nitroblue tetrazolium (27).

**Cell Growth**—Cell growth was assayed by measuring MTT reductase activity as described previously (18, 28). In the experiment depicted in Fig. 6, growth was assessed by counting cells in individual colonies at different times after plating at low density.

**Protein Determination**—Protein concentrations in cell homogenate supernatants were determined using the bicinchoninic acid method (29).

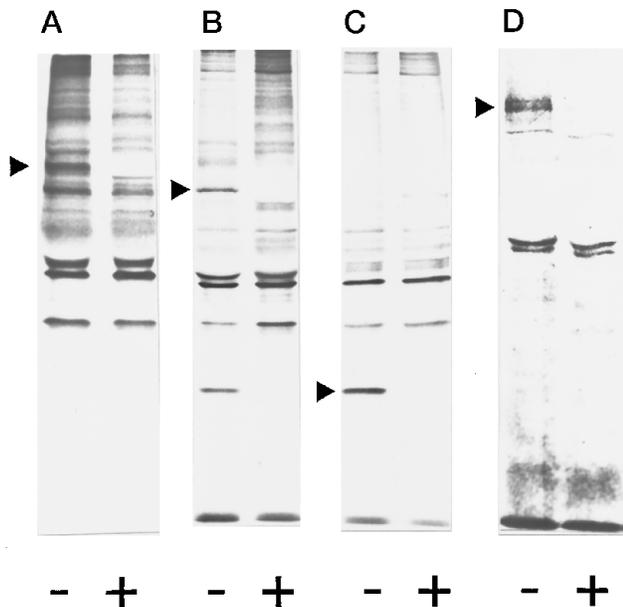
**Statistical Analysis**—Where *p* values are indicated, they were computed using Student's *t* test for non-paired variables.

## RESULTS

**Stability of Calpains in WI-38 Fibroblasts**—Human WI-38 fibroblasts were pulsed with [<sup>35</sup>S]Met and chased with nonradioactive Met as described under "Experimental Procedures." Control experiments showed that the antibodies against m-calpain and  $\mu$ -calpain large subunits, and the small subunit which is present in both calpain isozymes, quantitatively immunoprecipitated their respective antigens from cell lysates under the standard conditions (not shown). The identities of the <sup>35</sup>S-labeled calpain bands were ascertained by competitive displacement with purified, nonlabeled antigen (Fig. 1). Densitometric scanning of autoradiograms indicated that each of the <sup>35</sup>S-calpain subunits had relatively long apparent metabolic half-lives. The three subunits had very similar or identical intracellular stabilities; analysis of the data presented in Fig. 2 indicated apparent half-lives of 4.6, 5.2, and 4.6 days for m-calpain large subunit,  $\mu$ -calpain large subunit, and calpain small subunit, respectively.

Further experiments were designed to investigate the possibility that calpains might be less stable under some culture conditions. Therefore, cells were cultured for 48 h, at which time there was a small but detectable loss of <sup>35</sup>S label under routine culture conditions (Fig. 2). Conditions that destabilize calpain(s) should result in a significant decrease in residual <sup>35</sup>S label at this time. Similar rates of loss of <sup>35</sup>S label were obtained whether the cells were subconfluent (50% confluent at the time of pulse labeling) or 90% confluent (Table I). In most of our experiments, protein synthesis was allowed to continue during the chase interval. Thus, it could be argued that unlabeled calpain biosynthesized over the course of the experiments could result in an underestimation of the rate of loss of <sup>35</sup>S-calpain, by competing for degradation. To investigate this possibility, the disappearance of <sup>35</sup>S label in m- and  $\mu$ -calpain large subunits was measured in cells exposed to 100  $\mu$ M cycloheximide to inhibit protein synthesis during the chase period. No apparent acceleration of calpain degradation was detected in these studies (Table I). If calpain turnover is the result of autoprolysis, then calpain inhibitors should decrease the rate of loss of <sup>35</sup>S-labeled calpain. Addition of the cell-permeant calpain inhibitor ZLLY-CHN<sub>2</sub> during the chase period did not decrease <sup>35</sup>S-calpain loss (Table I).

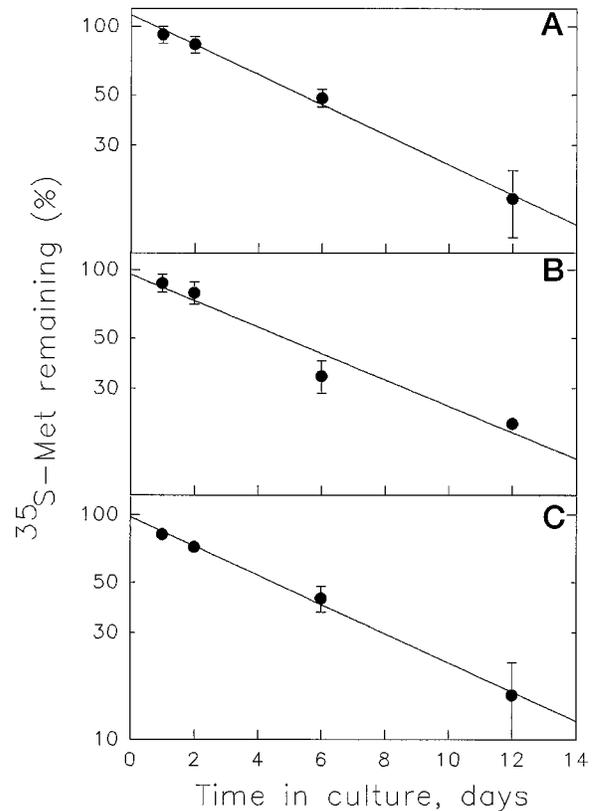
**Calpain and Calpastatin Stability in Human Cell Lines**—To determine whether the slow metabolic turnover of calpains was



**FIG. 1. Immunoprecipitation of calpains from WI-38 fibroblasts and calpastatin from C-33A cells.** Calpains or calpastatin were immunoprecipitated from  $^{35}\text{S}$ -labeled cells and subjected to electrophoresis and autoradiography as described under "Experimental Procedures." This figure presents a composite of several immunoblots each containing an antigen and the appropriate control lane. For competitive inhibition of  $^{35}\text{S}$ -antigen immunoprecipitation, 20  $\mu\text{g}$  of purified unlabeled human erythrocyte  $\mu$ -calpain, bovine myocardial m-calpain, or human erythrocyte calpastatin were added to the appropriate cell lysates prior to addition of the antibodies (lanes marked with a plus). *A*,  $\mu$ -calpain large subunit. *B*, m-calpain large subunit. *C*, calpain small subunit, with human erythrocyte calpain added to the control sample. *D*, calpastatin. Arrowheads mark the position of antigen bands, as determined by immunostaining of pure antigens.

unique to the WI-38 fibroblasts, several human cell lines were analyzed. Cervical carcinoma-derived HeLa cells, A-431 epidermoid carcinoma cells, SV-40 transformed lung epithelial cells (TE2 cells), and SV-40 transformed WI-38 cells (VA-13 cells) all displayed a similar small percentage loss of  $^{35}\text{S}$ -labeled calpains per 48 h (Table II). TE2 cells and C-33A cervical carcinoma cells were also analyzed for loss of  $^{35}\text{S}$ -labeled calpastatin. Although C-33A cells appeared to catabolize calpastatin more rapidly than TE2 cells, it was clear that calpastatin was relatively stable in either cell line, having a metabolic half-life measured in days, at least (Table II).

**Designing an Antisense Strategy to Deplete Calpain Small Subunit**—Because calpains appear to be slowly catabolized, experiments designed to decrease calpain content by administration of AS-ODNs to cultured cells were carried out over the course of days. Experiments with WI-38 and HeLa cells demonstrated the necessity for chronic exposure to the calpain AS-ODN in order to significantly decrease calpain small subunit content (Fig. 3). WI-38 cells cultured for 6 days in the presence of calpain AS-ODN displayed decreased calpain small subunit immunoreactivity (Fig. 3, panel A, lanes 6 and 7) compared with untreated cells. Consistent with the relative metabolic stability of calpains, treatment for 1 day did not decrease immunoreactivity (Fig. 3, panel A, compare lanes 2 and 3). Treatment with a control antisense oligodeoxynucleotide directed against calpastatin decreased calpastatin content in WI-38 cells (Fig. 3, panel A, lanes 10 and 11) but did not significantly alter the content of calpain small subunit (Fig. 3, panel A, lanes 8 and 9). Prolonged treatment of HeLa cells with calpain AS-ODN also resulted in substantial decrease in calpain small subunit content (Fig. 3, panel B); and again, this effect was not noticeable after 24 h exposure to the calpain



**FIG. 2. Time course of disappearance of label from calpain subunits in  $^{35}\text{S}$ Met pulse-labeled WI-38 cells.** WI-38 cells were labeled with  $^{35}\text{S}$ Met and then placed in medium containing nonlabeled Met. At various times afterward, calpains were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described under "Experimental Procedures." The amounts of calpain are expressed as percent of radioactive calpain band at day 0, on the basis of densitometric scans of the autoradiograms. Where shown, error bars indicate means  $\pm$  S.D. of three independent experiments. Data points without error bars are averaged values for two independent experiments. A linear regression line is included for each plot. *Panel A*, m-calpain large subunit; *panel B*,  $\mu$ -calpain large subunit; *panel C*, calpain small subunit.

**TABLE I**  
Metabolic stability of calpains in WI-38 cells under various culture conditions

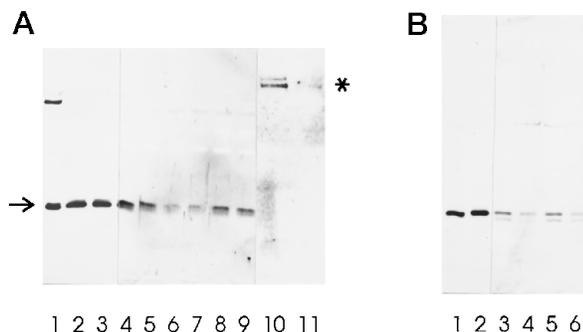
Condition	Percent decrease in $^{35}\text{S}$ label in 48 h	
	m-Calpain	$\mu$ -Calpain
90% confluent cells	24	23
50% confluent cells	23	13
Cycloheximide, 100 $\mu\text{M}$	16	13
ZLLY-CHN <sub>2</sub> , 20 $\mu\text{M}$	18	29

**TABLE II**  
Metabolic stability of calpains and calpastatin in human cell lines

Cell line	Percent decrease in $^{35}\text{S}$ label in 48 h		
	m-Calpain	$\mu$ -Calpain	Calpastatin
A-431	13	12	
HeLa	18	20	
VA-13	14	30	
TE2	24		10
C-33A			38

AS-ODN (Fig. 3, panel B, lanes 1 and 2). The control ODN for this experiment was a random ODN with a base composition identical to the calpain AS-ODN.

**Treatment with Calpain AS-ODN Resulted in Decreased Cell Growth**—Chronic exposure of WI-38 fibroblasts or HeLa cells

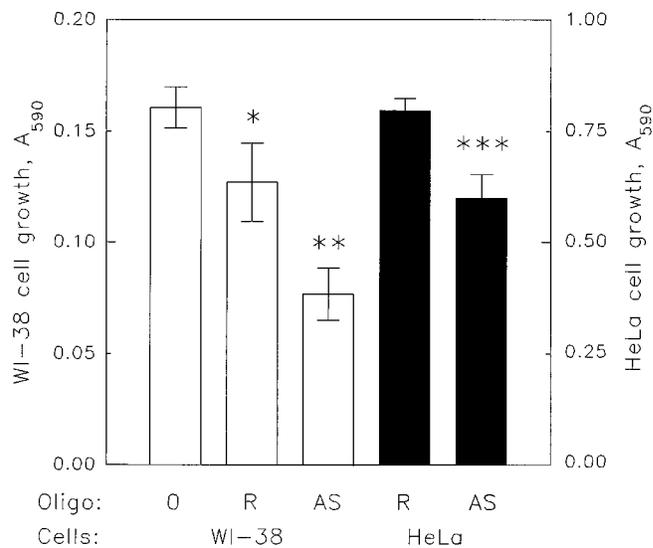


**FIG. 3. Depletion of calpain small subunit by calpain AS-ODN.** Panel A, WI-38 cells were cultured in 6-well plates and treated with ODNs for 1 day (lanes 2 and 3) or 6 days (lanes 4-11), followed by immunoblot analysis using P-1 monoclonal antibody against calpain small subunit. Immunoblotting was performed separately for the two time points, and the immunoblots were combined to produce this figure. Lane 1, purified calpain, 100 ng; lane 2, nontreated cells; lane 3, cells treated with calpain AS-ODN; lanes 4 and 5, nontreated cells; lanes 6 and 7, cells treated with calpain AS-ODN; lanes 8 and 9, cells treated with calpastatin AS-ODN. Lanes 10 and 11 were immunostained for calpastatin: lane 10, nontreated cells; lane 11, treated with calpastatin AS-ODN. Protein load for all WI-38 samples was 6.6  $\mu$ g. The arrow indicates calpain small subunit, and the asterisk indicates calpastatin. Panel B, HeLa cells were treated with ODNs as described above for 1 day (lanes 1 and 2) or 6 days (lanes 3-6). Lane 1, nontreated; lanes 2, 4, and 6, calpain AS-ODN; lanes 3 and 5, random ODN. Gel loads were normalized on the basis of MTT reductase activity in the HeLa cells prior to homogenization.

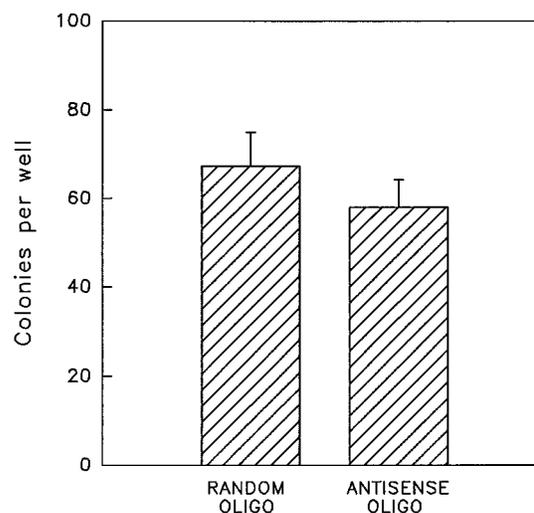
to 0.2  $\mu$ M calpain AS-ODN in the culture medium led to decreased cell growth, relative to samples in other wells of the same culture plate treated with the random ODN control (Fig. 4). One or two days of exposure did not produce significantly altered growth rates. For example, treatment of HeLa cells for 48 h with calpain AS-ODN gave MTT reductase growth assay results of  $0.304 \pm 0.019 A_{590}$  units, whereas treatment with the random oligo produced an  $A_{590}$  of  $0.315 \pm 0.002$ . This is consistent with the assumption that pre-existing calpain must be metabolically cleared before an effect on growth can be observed. Since it was possible that the calpain AS-ODN produced more toxicity than the random ODN, resulting in less apparent cell growth, we investigated the chronic toxicities of these two ODNs by a clonal survival assay (Fig. 5). Calpain AS-ODN was not significantly more toxic than the random ODN ( $p = 0.17$ ).

Cells were plated at relatively high density to utilize the MTT reductase assay for cell growth and to harvest a sufficient number of cells for immunoblot analysis. Although statistically significant (Fig. 4), the effect of calpain AS-ODN on cell growth was not dramatic under these conditions, mainly because the control cells were approaching confluence by the end of the experiments and therefore growing slowly. There were other indications that the calpain AS-ODN-treated cells were growth-arrested. When parts of the culture wells were scraped to denude the substrate, WI-38 fibroblasts or HeLa cells treated with calpain AS-ODN for 7 days did not fill in these areas after several days of further culture, while cells exposed to random ODN controls completely overgrew the scraped sites (not shown).

Optimum conditions for observation of reduced growth rate by calpain AS-ODN treatment might be expected at very low seeding densities. This would prevent the control cells from approaching confluence prior to the end of the experiment. Therefore, another protocol was devised for assessing inhibition of cell growth by calpain AS-ODN. HeLa cells were plated at low density, and the effects of calpain AS-ODN and the random ODN on clonal cell growth were assessed by directly counting cells in colonies. Within a few days of treatment, the



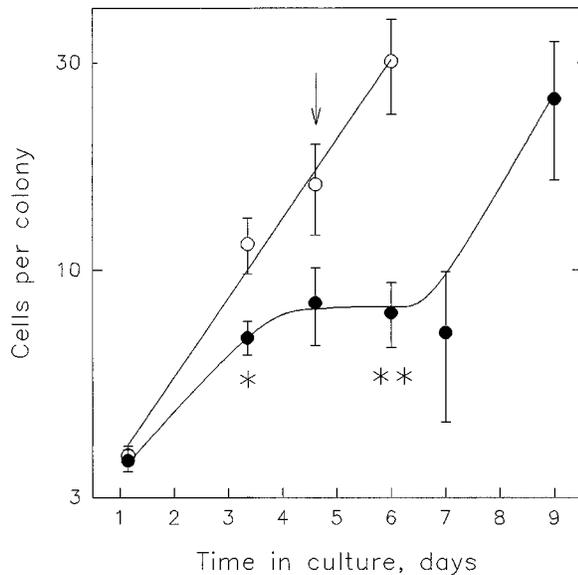
**FIG. 4. Inhibition of WI-38 cell growth by addition of calpain AS-ODN to the culture medium.** WI-38 cells were 20-30% confluent at the beginning of the study and were treated as described under "Experimental Procedures" with no ODN (O), random ODN (R), or calpain AS-ODN (AS) for 12 days. HeLa cells were treated with ODNs for 6 days and were 50% confluent at the beginning of the experiment. At the end of the experiments, cell growth was assessed by the MTT reductase method (\*,  $p = 0.01$  versus no ODN; \*\*,  $p = 0.014$  versus R; \*\*\*,  $p = 0.005$  versus R).



**FIG. 5. Clonal survival assay for assessing the relative toxicity of calpain AS-ODN.** HeLa cells were cultured for 10 days in the presence of calpain AS-ODN or the random ODN and then subcultured in DMEM containing 10% bovine serum in 35-mm dishes at a density of 100 cells per  $\text{cm}^2$ . After 5 days in culture, viable cells were stained with MTT, and colonies were counted. The data presented are means  $\pm$  S.D. of three culture dishes for each condition.

calpain AS-ODN-treated colonies stopped growing after attaining an average size of eight cells (Fig. 6, filled circles). The inhibition of growth was reversible upon removal of calpain AS-ODN from the culture medium (arrow in Fig. 6). However, there was a lag of several days before cell proliferation resumed. A similar delayed recovery has been observed in several cell lines following depletion of calpain activity with the cell-permeant, irreversible calpain inhibitor, ZLLY-CHN<sub>2</sub>.<sup>2</sup> The random ODN-treated cells continued to grow in the presence of the oligonucleotide (Fig. 6, open circles).

<sup>2</sup> R. L. Mellgren, unpublished observations.



**FIG. 6. The effect of calpain AS-ODN on clonal growth of HeLa cells.** HeLa cells were plated in a 48-well culture plate at a density of approximately 30 cells/well. Calpain AS-ODN was added to 12 wells, and random ODN to another 12 wells. After 28 h the wells were examined for colonies, and a total of 23 and 20 colonies, respectively, were detected in the calpain AS-ODN and random ODN samples. Control wells plated and cultured without ODNs had approximately four times the number of colonies, indicating that both ODNs had a similar negative effect on colony formation. At various times after plating, the number of cells/colony  $\pm$  S.E. was determined (\*,  $p = 0.010$  versus random ODN at same time point; \*\*,  $p = 0.0067$  versus random ODN at same time point). Open circles, random ODN; filled circles, calpain AS-ODN. After 4.7 days, both ODNs were removed from the culture medium (arrow).

#### DISCUSSION

It has become apparent in recent years that intracellular proteolysis has a key role in regulating some signal transduction pathways and cell cycle progression (reviewed in Ref. 30). Most of these regulated proteolytic events have been ascribed to the ubiquitin-dependent proteolytic system. However, one would predict that the calpains are involved in these types of events as well, given their absolute requirement for the intracellular messenger  $\text{Ca}^{2+}$  and their structural similarity to the universal  $\text{Ca}^{2+}$ -signal transducing protein, calmodulin. Studies of the role of calpains in cell proliferation (17, 18) have suffered from the necessity of relying on less than ideal inhibitors to block calpain activity in cells. The most specific cell-permeant calpain inhibitors available at this time have inhibitory activity on other known proteases (31–33). Even when appropriate controls are utilized to minimize the possibility of interactions with other target proteases, one can always argue that a calpain inhibitor's influence on cell function could be explained by interaction with an as yet unrecognized target. To address this problem, we have established a method for depletion of intracellular calpain small subunit by chronic exposure of cells to a calpain AS-ODN-LipofectAMINE complex in the cell culture medium.

In preparation for studying calpain AS-ODN effects on cell growth, the time course for disappearance of  $^{35}\text{S}$ -labeled calpains was investigated under routine culture conditions. In light of the hypothesis that calpains are activated in cells by autoproteolysis (1, 19), the observation that the major calpain isozymes displayed considerable metabolic stability was surprising. The apparent lack of an effect of the calpain inhibitor ZLLY-CHN<sub>2</sub> on metabolic stability (Table II) indicated that even the small loss of calpain over 48 h was not the result of autoproteolysis. These observations suggest the need to re-

evaluate the notion that calpains must undergo autoproteolysis in order to become activated. Indeed, as this work was in progress, a study was published indicating that calpain autoproteolysis is not required for cleavage of rat erythrocyte membrane proteins (21). An alternative interpretation of our results is that calpains are not chronically active but only become activated (perhaps by autoproteolysis) under infrequently occurring or transitory intracellular conditions.

Within the context of the present work, the metabolic stability of calpains indicates that chronic exposure of cells to calpain AS-ODN would be necessary in order to deplete calpains. That this is the case for WI-38 and HeLa cells is shown in Fig. 3. It was also important to present calpain AS as a complex with the cationic detergent (LipofectAMINE); treatment of WI-38 cells and various human cell lines for 1 week with 0.2–10  $\mu\text{M}$  calpain AS-ODN in the absence of LipofectAMINE did not deplete calpain small subunit (not shown). When added to culture medium as a complex with LipofectAMINE, 0.2  $\mu\text{M}$  calpain AS-ODN effectively depleted calpain and inhibited cell growth.

The results of this study are consistent with the notion that calpain depletion produces inhibition of cell growth in WI-38 human diploid fibroblasts and HeLa cells. Calpain AS-ODN caused depletion of calpain small subunit in both these cell lines, whereas a calpastatin AS-ODN and the random ODN did not (Fig. 3). At the same time, cell growth was specifically decreased by calpain AS-ODN (Figs. 4 and 5). This was most readily apparent in cells plated at low density (Fig. 6). In addition, this latter study demonstrated that growth inhibition was reversed by removal of the calpain AS-ODN and was consequently not an irreversible toxic effect.

Depletion of calpastatin by AS-ODN (Fig. 3, panel A, lane 11) might be expected to increase cell proliferation, since calpastatins are specific inhibitors of calpains. In fact, this was found not to be the case, and a modest inhibition of WI-38 cell growth was observed (not shown). Inhibition by calpastatin AS-ODN was always less than with calpain AS-ODN and similar to the inhibition of WI-38 cell growth observed for the random ODN (Fig. 4). Calpains and calpastatins appear to be localized at different intracellular sites (26). Thus, it is possible that calpastatins do not interact with calpains within the context of cell growth regulation.

Previous studies have indicated that calpain-like proteases participate in regulation of cell growth (16–18). However, these investigations have utilized cell-permeant protease inhibitors of varying specificity to block intracellular calpain activity. The most specific calpain inhibitor utilized for this purpose has been ZLLY-CHN<sub>2</sub>. Radioiodinated ZLLY-CHN<sub>2</sub> has been shown to specifically label calpain large subunit upon exposure of platelets to the  $\text{Ca}^{2+}$  ionophore, A23187 (32, 33). In addition, cathepsin L forms appear to be labeled in a  $\text{Ca}^{2+}$ -independent manner. By using a cathepsin L-selective inhibitor as a control, it was possible to present evidence that a ZLLY-CHN<sub>2</sub>-sensitive target, presumably a calpain or calpain-like protease, was necessary for proliferative growth of TE2 and C-33A cells, and a number of other human, rat, and hamster cell lines (18). The present work indicates that a target for an AS-ODN designed to complement calpain small subunit mRNA also specifically inhibited the growth of WI-38 fibroblasts and HeLa cells. Taken together, these studies provide strong evidence that calpains are required for proliferative growth of mammalian cells. Future investigations will focus on delineating the specific roles of the major calpain isozymes in the cell division cycle.

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