Calpains are intracellular Ca\(^{2+}\)-dependent proteases that are thought to participate in 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 \(^{35}\)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 isoforms, 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 Ca\(^{2+}\)-requiring intracellular cysteine proteases that are widely, perhaps universally, distributed in cells of multicellular animals (1–5). All mammalian cell studied thus far appear to contain one or both of the two major calpain isoforms, m-calpain, which requires millimolar Ca\(^{2+}\) for activity in extracts, and \(\mu\)-calpain, which requires 10–100 \(\mu\)M 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 isoform 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 Ca\(^{2+}\)-dependent signal transduction pathways. They possess calmodulin-like 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 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\(^{35}\)S-Label\(^{TM}\), metabolic labeling reagent containing 70% of the label as \(^{35}\)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 agarose was obtained from Pierce. Phosphorothioate AS-ODNs were synthesized by Oligos Etc. Calpain AS-ODN (AGGAACATGGCTGCGACTC) 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 (CGGACGCAACACTGGAAGA) was antisense to the initiation Met regions of human calpain small subunit and human calpastatin mRNAs, respectively. A “random” phosphorothioate ODN (CGGACGCAACACTGGAAGA) 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 sequences reported in this paper has been submitted to the GenBank\(^{TM}\) and EBI Data Bank with accession number(s) X04366 and X04106.

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§ 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; ZL-L-CH\(_2\)N\(_2\), benzylxoxycarbonyl-Leu-Leu-Tyr diazomethyl ketone; Mops, 4-morpholinoethanesulfonic 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$_2$ 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 the presence of the isozyme (25). A mouse monoclonal antibody developed against human μ-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 m-calpain large subunit in immunoblots (26). Preliminary studies showed that it did not efficiently immunoprecipitate purified human erythrocyte m-calpain under the conditions used in our studies, but it recognized calpain small subunit in protein immunoblots. Calpastatin was immunoprecipitated with P-1 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 μCi of [35S]Met was added. Cells were labeled for 12 h and then placed in the usual culture medium, which contained 0.2 ml nonradioactive Met. At zero time, and various times after removal of the [35S]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 μM leupeptin, and 10 μM phenylmethylsulfonyl fluoride, pH 7.5). The cell lysate was incubated on ice for 30 min and centrifuged at 10,000 × g for 20 min at 4 °C. To the resultant supernatant, SDS was added. The supernatant was heated at 95°C for 5 min and centrifuged to separate eluted proteins from the cell lysate. Two- to four-fold diluted Sds-sample buffer was added, and the samples were heated to 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 35S-labeled protein bands. A standard curve for estimating relative radioactivity was prepared by precipitation of [35S]Met into a chilled vial and centrifugation. The percentage of [35S]Met remaining in the supernatant was determined using the bicinchoninic acid method (29).

Stability of Calpains in WI-38 Fibroblasts—Human WI-38 fibroblasts were pulsed with [35S]Met and chased with nonradioactive Met as described under “Experimental Procedures.” Control experiments showed that the antibodies against m-calpain and μ-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 35S-labeled calpain bands were ascertained by competitive displacement with purified, nonlabeled antigen (Fig. 1). Denitritometric scanning of autoradiograms indicated that each of the 35S-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, μ-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 35S label under routine culture conditions (Fig. 2). Conditions that destabilize calpain(s) should result in a significant decrease in residual 35S label at this time. Similar rates of loss of 35S 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 35S-calpain, by competing for degradation. To investigate this possibility, the disappearance of 35S label in m- and μ-calpain large subunits was measured in WI-38 cells exposed to 100 μM 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 autoproteolysis, then calpain inhibitors should decrease the rate of loss of 35S-labeled calpain. Addition of the cell-permeant calpain inhibitor ZLLY-CHN$_2$ during the chase period did not decrease 35S-calpain loss (Table I).

Calpain and Calpastatin Stability in Human Cell Lines—To determine whether the slow metabolic turnover of calpains was
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 35S-labeled calpains per 48 h (Table II). TE2 cells and C-33A cervical carcinoma cells were also analyzed for loss of 35S-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 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...
Calpains Are Long-lived Proteins

Panels A and B, 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 μg. 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 μM 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 ± 0.019 A590 units, whereas treatment with the random oligo produced an A590 of 0.315 ± 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 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 permeant, irreversible calpain inhibitor, ZLLY-CHN2.2 The random ODN-treated cells continued to grow in the presence of the oligonucleotide (Fig. 6, open circles).

**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 μg. 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.

**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 cm². After 5 days in culture, viable cells were stained with MTT, and colonies were counted. The data presented are means ± S.D. of three culture dishes for each condition.

R.L. Mellgren, unpublished observations.
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 μM 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 μM 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-CHN2. Radiiodinated ZLLY-CHN2 has been shown to specifically label calpain large subunit upon exposure of platelets to the Ca2+ ionophore, A23187 (32, 33). In addition, cathepsin L forms appear to be labeled in a Ca2+-independent manner. By using a cathepsin L-selective inhibitor as a control, it was possible to present evidence that a ZLLY-CHN2-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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