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Calpain Inhibition Decreases the Growth Rate of Mammalian Cell Colonies*

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The calpains, a family of calcium-requiring intracellular proteases, are proposed regulators of cell proliferation. However, ablation of the calpain small subunit gene necessary for function of the conventional calpains did not result in decreased rate of proliferative growth of mouse stem cells under routine culture conditions. To address the reasons for this discrepancy, Chinese hamster ovary cell lines were established that overexpress the calpain inhibitor protein, calpastatin, under control of the ecdysone congener, ponasterone A. Overexpression of calpastatin in these cell lines resulted in a decreased growth of isolated colonies adhering to tissue culture plates. However, when cells were plated at higher density, calpastatin overexpression had no influence on proliferative growth rate. Growth of colonies in soft agar was not inhibited by calpastatin overexpression. Cell adhesion, cell de-adhesion, and cell motility all appeared to be normal after calpastatin overexpression. Differential display analysis was initiated to detect possible alteration of gene expression upon calpastatin overexpression. Analysis of ~3000 differential display PCR signals resulted in identification of one band that was underexpressed. Northern blot analysis confirmed a decreased amount of ~1 kb mRNA in cells overexpressing calpastatin. Sequence analysis identified a putative protein, Csr, containing a region homologous to two ubiquitin transferases and a putative cation channel protein.

The calpains comprise a large family of intracellular cysteine proteases (1, 2). Various members of the calpain protease family participate in signal transduction pathways as diverse as adaptation of yeast to an alkaline environment (3), nematode sex determination (4), and development of the optic lobes in *Drosophila* (5). The conventional calpains, m- and μ-calpain, are ubiquitously expressed, require calcium ion for activity, and are subject to regulation in most cells by an endogenous inhibitor protein, calpastatin.

Very recently, a number of studies have shown that calpains are important regulators of cell-substratum interactions and cell motility (6–10). Rear cell detachment and migration of CHO[1] cells was shown to be inhibited by cell permeable calpain inhibitors (7). These studies indicated that calpains are required for remodeling or weakening of the cytoskeleton-integrin linkage to allow efficient rear detachment during migration. Other studies have suggested a role for calpains in integrin clustering necessary for T-cells to bind to ICAM-1 (intercellular adhesion molecule 1) (9). Cell attachment to substratum and subsequent spreading may also require participation of calpains. The spreading of NIH-3T3 fibroblasts on culture plates was inhibited by calpeptin and several other calpain inhibitors (11). It was also inhibited in cells transfected with calpastatin. Recently, EGF activation of m-calpain in rat kidney fibroblasts has been reported (8). IP-10 partially prevented calpain activation in response to EGF (12). It also counteracted EGF's effect on fibroblast detachment from substrate and migration.

Calpains may influence cell proliferation. Calpain inhibitors have potent anti-mitogenic effects (13, 14), and μ-calpain antisense oligonucleotides were shown to decrease growth of CHO cell colonies (15). Calpains appear to be necessary for the clonal growth of pre-adipocytes that is required prior to adipocyte differentiation (16, 17). However, the involvement of calpains in cell proliferation is controversial. Many of the cell-permeable inhibitors used in previous studies are not absolutely specific for calpains. Moreover, mouse *Capn4*$^{(-/-)}$ stem cells, which do not express the small subunit present in m- and μ-calpain, grow at the same rate as *Capn4*$^{(+/+)}$ cells (18).

In the present study, CHO cell lines that express calpastatin under control of the insect hormone, ponasterone A, were utilized to address the role of calpains in proliferative cell growth. We present evidence that calpains do contribute to proliferation of CHO cells and mouse fibroblasts, but only when the cells are growing in isolated colonies attached to a growth surface.

EXPERIMENTAL PROCEDURES

Materials

Cell Culture—EcR-CHO cells were obtained from Invitrogen. *Capn*4 homozygous knockout cells (*Capn4*$^{-/-}$) and wild-type cells (*Capn4*$^{+/+}$) were kindly supplied by Dr. John Elce (Queen's University, Canada). DMEM and HF-12 growth media were obtained from Mediatech. FBS was from HyClone. Trypsin-EDTA (10×) was obtained from Sigma Chemical Co. Geneticin was purchased from Cellgro. Zeocin was from Invitrogen. Cell culture plates, dishes, and flasks were purchased from Corning. Radioisotopes were obtained from ICN.

Molecular and Cell Biology—Human full-length calpastatin cDNA was a generous gift from Dr. Masatoshi Maki (Nagoya University). Restriction endonucleases, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Promega. DH5α competent *Escherichia coli* were from Invitrogen. Bacto-tryptone and yeast extract for LB medium were obtained from Difco. Ponasterone A and pIND (SP1)/V5-His C mammalian expression vector were purchased from Invitrogen. Nucleic acid mass markers, 1-kb DNA ladder, 100-bp DNA ladder,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF484417.

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[1] The abbreviations used are: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; MTT, (3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; EGF, epidermal growth factor; FLC, full-length calpastatin; IP-10, interferon inducible-protein 10.

and 0.24- to 9.5-kb RNA ladder, were supplied by Invitrogen. A QIAquick PCR Purification kit, a QIAquick Gel Extraction kit, and a QIAprep Spin Miniprep kit were purchased from Qiagen.

Western Blotting—Electrophoresis supplies and nitrocellulose membranes used in immunoblotting were obtained from Bio-Rad. Alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG), 3-indolyl phosphate, and nitro blue tetrazolium were purchased from Sigma Chemical Co. Human erythrocyte calpastatin was purified to near homogeneity by established procedures (19). Antibodies used for Western blotting were: mouse monoclonal antibody 5-8A, which was developed against human calpastatin and did not cross-react with hamster calpastatin, and mouse monoclonal antibody P-1, which recognizes the small subunit common to both μ- and m-calpain.

Methods

Cell Culture—EcR-CHO cells and derived transfected cell lines were cultured in HF-12 medium containing 10% FBS. The FLC-EcR-CHO cell lines were established by transfecting pIND (SP1)/V5-His vector containing human full-length calpastatin (FLC) cDNA into EcR-CHO cells by electroporation. Mock transfected control cells were obtained by transfecting the vector alone into EcR-CHO cells. FLC-EcR-CHO and mock transfected cells were cultured in selection medium containing 1.4 mg/ml Geneticin and 250 μg/ml Zeocin every four to five passages. *Capn4*$^{(+/+)}$ and *Capn4* $^{(-/-)}$ cells were cultured in DMEM containing 10% FBS. Unless otherwise indicated, all cells were cultured as monolayers at 37 °C and 5% CO_2. In some experiments, cells were cultured suspended in HF-12 medium plus 10% fetal calf serum containing 0.75% agar.

Electrophoresis and Protein Immunoblotting—Cells were trypsinized and washed twice with HBSS, and the cell number was determined using a Coulter cell counter. Approximately 80,000 cells were transferred to a new tube and centrifuged to form a pellet. The supernatant was removed by aspiration, and 80 μl of sterile H_2O and 40 μl of SDS-PAGE sample prep buffer were added and mixed with the cell pellet by pipetting. The sample was heated at 100 °C for 5 min, and 40 μl was applied to 10% polyacrylamide gels.

Protein Determination—Protein concentrations in cell homogenate 10,000 $\times$ *g* supernatants were determined using the BCA method (20) according to the manufacturer's instructions.

Calpain Assay—Cells were grown in T75 flasks to confluence, washed three times with 37 °C HBSS, and lysed with 0.4 ml of ice-cold lysis buffer I (pH 7.4): 50 mM imidazole-HCl, 50 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 10 μM pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. Cell lysates were scraped off the flasks and homogenized for ~8 strokes. After removing the cell debris by centrifugation for 10 min at 10,000 $\times$ *g* at 4 °C, 25 μl of supernatant was assayed for calpain activity, using ^{14}C-methylated casein as the substrate (21).

Calpastatin Purification and Assay—To partially purify calpastatin, cell pellets were homogenized in lysis buffer II (50 mM imidazole-HCl, 200 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100, pH 7.4) by passage through pipette tips several times. Because calpastatin is known to be resistant to heat denaturation, it was partially purified by heat treatment of the homogenate at 100 °C for 10 min and centrifuging at 14,000 $\times$ *g* for 10 min to remove denatured proteins. Only the supernatant fraction contained calpastatin activity. Calpastatin activity was determined by its ability to inhibit a defined amount of purified human erythrocyte μ-calpain (1 ng/μl) in the standard ^{14}C-caseinolytic assay.

Cell Growth Assays—Proliferative cell growth was measured directly by counting suspended trypsinized cells from 6-well plates using a Coulter cell counter. At least two counts were taken for each sample, and the average was taken for calculating the cell number. In some experiments, cell growth was estimated by measurement of MTT reductase activity as described previously (22).

Cell Attachment Assay—Cells were trypsinized at confluence, washed with HF-12 medium once, and resuspended in fresh HF-12 medium containing 10% FBS. The cells were added at a concentration of 10^5 cells/ml to 6-well plates at 37 °C and then observed at 30, 60, and 120 min using inverted phase-contrast microscopy.

Cell Detachment Assay—Cell detachment was assessed (*a*) by measuring the detached cell number in the supernatant after 5 min of shaking (power level 5) of the culture plates on a Mini-Orbital Shaker (BellCo); and (*b*) by measuring the adherent cell number after successive PBS washes as described previously (23), with minor modifications. Briefly, cells were allowed to adhere and spread in serum-containing medium to 75–85% confluence. Medium was then carefully removed, and cells were gently washed three times with Ca^{2+}- and Mg^{2+}-free PBS for 5 min/wash. Wash solutions were then discarded and the cells remaining on the plate were trypsinized and counted. Percent detachment was calculated by comparison with control plates that were not washed.

Cell Motility Assay—Cell migration was assessed by the ability of the cells to move into an adjacent area as described previously (24). Cells were plated at high dilution on a 6-well plate and grown as isolated colonies in the presence or absence of ponasterone A. Colonies were scratched with a sterile pipette tip to produce cell-free zones. The plates were then incubated at 37 °C for observation of cell migration into the denuded zones. Photographs were taken at 0, 12, and 24 h.

Differential Display—The Delta Differential Display kit (CLONTECH) was utilized for differential display analysis, using the manufacturer's directions throughout. The protocol is based on the original description of differential display analysis (25). Purified total RNA fractions from the cell lines utilized were treated with DNase to remove traces of genomic DNA that might have interfered with the display assays. Briefly, RNA was isolated from FLC-EcR-CHO clone 83 and mock transfected EcR-CHO cells that were cultured in the absence or in the presence of ponasterone A, giving a total of four samples for differential display. After DNase treatment, first-strand cDNA synthesis was carried out, and differential display PCR was performed using 90 combinations of the 5′ and 3′ primers supplied with the kit. The ^{33}P-labeled PCR bands were separated on a DNA sequencing gel, and autoradiograms were visually inspected for alterations in expression patterns in the four samples.

Bands that were selectively enriched or depleted in the clone 83 plus ponasterone sample were selected for PCR amplification, ^{32}P labeling, and use as probes in Northern blotting against poly(A)-RNA isolated from each of the four cell cultures. The Northern-verified positive band was extracted from the differential display gel, re-amplified by PCR using the same differential display primer set, and T/A-cloned into pT-Adv vector. TOP10F′ *E. coli* were transformed with the vector, and two positive clones were selected and amplified for vector isolation and sequencing of the insert.

Statistical Analysis—Data were analyzed by unpaired Student's *t* test. Differences in means were considered to be statistically significant if $p < 0.05$. *Error bars* in the graphs represent standard deviation.

RESULTS

Inducible Expression of Human Calpastatin in EcR-CHO Clones 83 and 106—Two EcR-CHO clones stably transfected with human full-length calpastatin were generated and isolated as described under "Methods." These cell lines are designated FLC-EcR-CHO clone 83 (clone 83) and FLC-EcR-CHO clone 106 (clone 106). Both were capable of expressing human calpastatin when cultured in the presence of 5 μM ponasterone A (Fig. 1*A*). Both of the clones also expressed small amounts of human calpastatin constitutively. Maximum calpastatin expression was observed after ~3–4 days of exposure to ponasterone (Fig. 1*B*). In other studies not shown, the calpastatin was shown to be expressed almost entirely in soluble form. Because we wished to use the calpastatin-expressing clones to study the effects of calpain inhibition on cell growth, it was important to monitor calpain content upon calpastatin overexpression. Protein immunoblot analysis indicated that calpain small subunit expression was not substantially altered by induced overexpression of calpastatin (Fig. 1*C*). Although the experiment depicted in Fig. 1 suggests a modest increase in calpain at the 96-h time point plus ponasterone, this was not reproducible in other experiments. Because both m- and μ-calpain depend on small subunit for activity, these results indicate that levels of functional calpain protein are not influenced by the degree of calpastatin overexpression achieved in our studies. Moreover, the content of μ-calpain large subunit did not change (data not shown), indicating that the balance of μ- and m-calpain was not altered.

To determine if the expressed calpastatin was capable of inhibiting calpain, heat-treated cell lysate fractions were prepared and assayed for calpastatin as described under "Methods." Both human calpastatin-expressing clones displayed in-

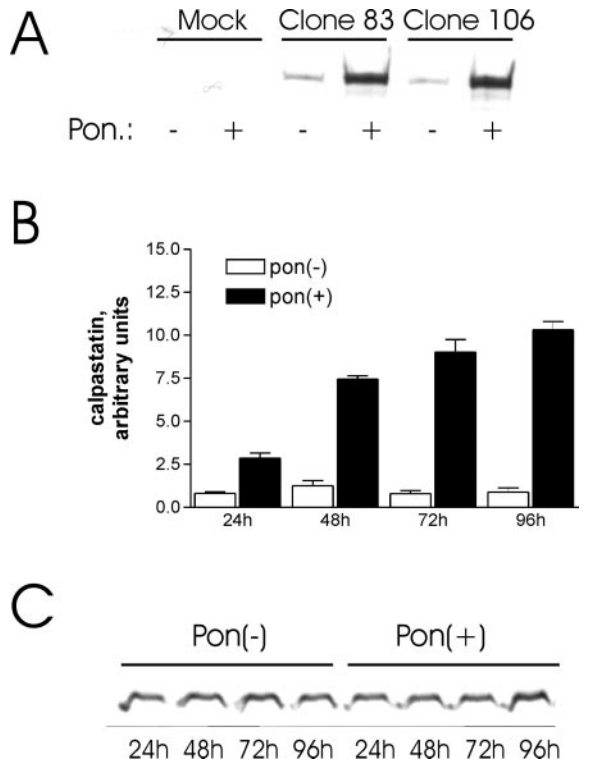

FIG. 1. **Inducible overexpression of calpastatin in EcR-CHO cells transfected with human full-length calpastatin.** *A*, mock transfected EcR-CHO cells, clone 83 cells, or clone 106 cells were cultured for 72 h with or without 5 μM ponasterone A in the culture medium. Cell lysates were prepared and subjected to protein immunoblotting using a human-specific calpastatin antibody. *B*, clone 83 cells were cultured with or without ponasterone A for the indicated time and processed for protein immunoblots utilizing human-specific anti-calpastatin. Triplicate blots were analyzed by densitometric scanning. Results are recorded as arbitrary density units. *C*, One set of the same samples used in *B* was immunoblotted using P-1 antibody against calpain small subunit as the first antibody.

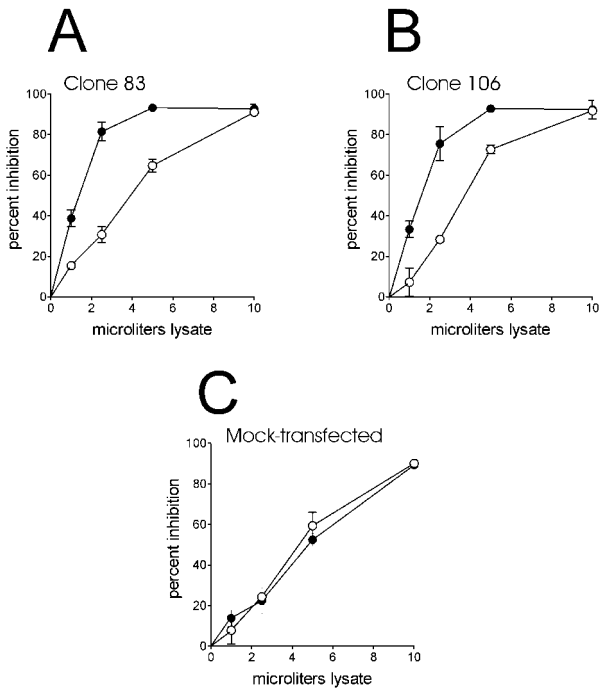

FIG. 2. **Increased calpastatin activity in ponasterone-induced clones 83 and 106.** Cell lines were cultured with (●) or without (○) ponasterone A for 72 h, then lysates were prepared and assayed for calpastatin activity as described under "Methods." *A*, clone 83; *B*, clone 106; *C*, mock transfected EcR-CHO cells.

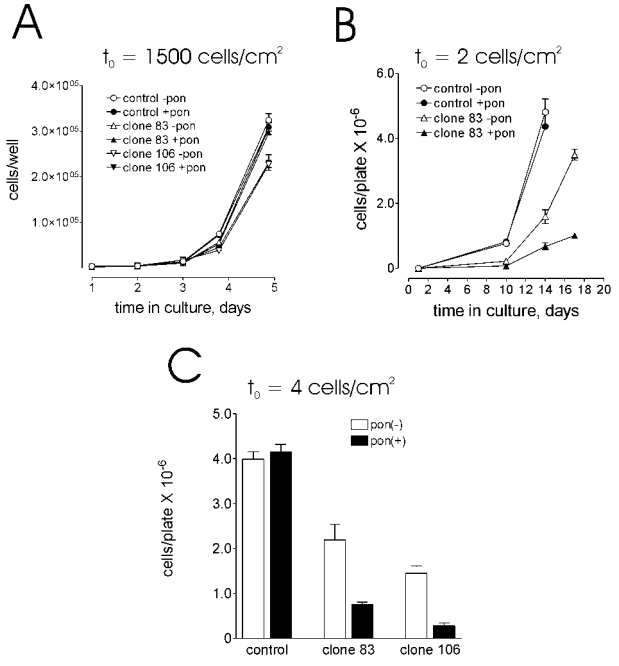

FIG. 3. **Growth of calpastatin-overexpressing clones.** *A*, cells were plated at a density of 1500 cells/cm^2 and cultured with or without 5 μM ponasterone A. *Circles*, mock transfected; *triangles*, clone 83; *inverted triangles*, clone 106. *Filled symbols*, plus ponasterone samples. *B*, clone 83 (*triangles*) and mock transfected cells (*circles*) were plated at 2 cells/cm^2 and cultured plus (*filled symbols*) or minus ponasterone A. *C*, in a separate experiment, mock transfected, clone 83, and clone 106 cells were plated at 4 cells/cm^2 and cultured for 15 days plus (*filled bars*) or minus ponasterone.

creased calpastatin activity in response to ponasterone, whereas the mock transfected cells did not (Fig. 2).

Effect of Calpastatin Overexpression on Clonal Cell Growth—There was no apparent effect of calpastatin overexpression on the growth of cells plated at cell densities as low as 1.5×10^3 cells/cm^2 (Fig. 3*A*). These results agree with previous studies on growth of *Capn4*$^{(-/-)}$ embryonic mouse stem cells (20) and do not support the notion that the conventional calpains are important for controlling cell proliferation. However, there are indications that calpain activity may be important for controlling clonal growth of cells (15, 17). To investigate this further, cells were plated at clonal densities (2–4 cells/cm^2). Under these conditions, induction of calpastatin expression resulted in a significant decrease in growth of colonies (Fig. 3, *B* and *C*). Ponasterone A had no detectable effect on clonal growth of the mock transfected EcR-CHO cells. There was no apparent difference in plating efficiencies of the clone 83, clone 106, and mock transfected cells, which varied in different experiments between 40 and 50%.

Clone 83 and 106 cells also grew more slowly than mock transfected cells in the absence of ponasterone (Fig. 3, *B* and *C*). It is tempting to speculate that this was the result of leakage of human calpastatin expression (Fig. 1*A*). However, in the absence of calpastatin overexpression, clones 83 and 106 contained nearly the same *total* calpastatin levels (*i.e.* human plus hamster) as the mock transfected cells (Fig. 2, compare minus ponasterone values in *panels A* and *B* with *panel C*). It may be that the splice form of human calpastatin utilized for transfection has unique growth inhibitory properties relative to endogenous hamster calpastatin forms. However, the slower basal growth rates could be intrinsic properties of the founder cells for clones 83 and 106. Further studies will be required to address this issue.

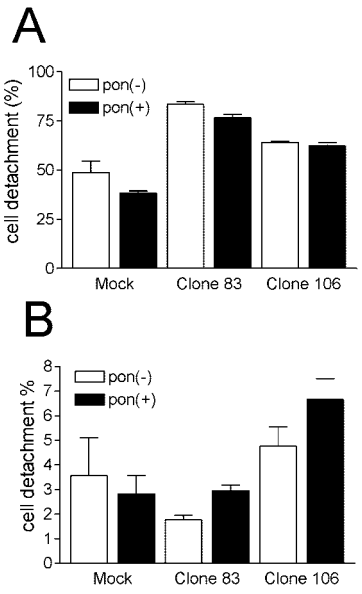

FIG. 4. **Calpastatin overexpression did not affect cell detachment.** Clone 83, clone 106, and mock transfected cells were cultured with or without ponasterone until about 50% confluent. *A*, cells were subjected to three successive PBS washes to detach cells as described under "Methods." *B*, cells were detached by mechanical agitation as described under "Methods."

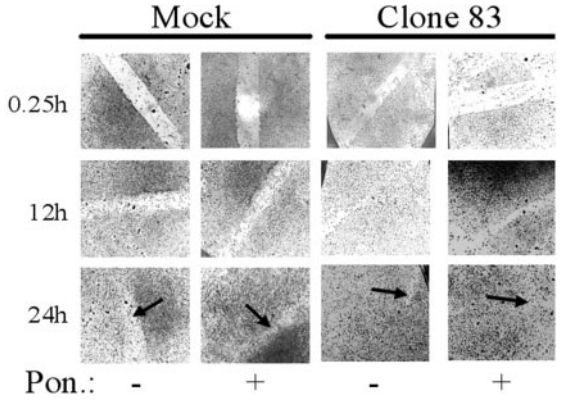

FIG. 5. **Calpastatin overexpression did not affect cell motility.** Colonies of clone 83 and mock transfected cells were grown with or without ponasterone A. A sterile pipette tip was utilized to produce a scratch across the colony, and growth of cells into the denuded zone was monitored by light microscopy. *Arrows* denote the nearly filled scratch zones at 24 h.

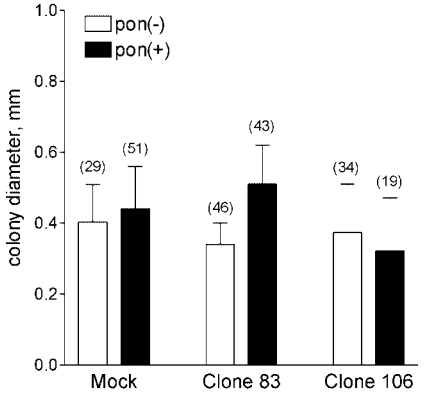

FIG. 6. **Calpastatin overexpression did not affect colony size in agar.** Cells were cultured at a density of 100/dish in 0.75% agar containing HF-12 medium with or without ponasterone A in 60-mm dishes scored with a 1-mm grid. After 12 days, the colonies were stained with 0.005% crystal violet, and photographed. The numbers of colonies measured for each condition are given in *parentheses above the graph bars*.

Morphologically, the clonal growth of calpastatin-overexpressing cells was characterized by symmetrical round colonies, with few cells growing between (not shown). This growth pattern might have reflected a decreased ability of calpastatin-overexpressing cells to detach from colonies, or a decreased ability to re-attach and grow between the colonies. Alternatively, the cells may not have been able to migrate on the substratum to populate the inter-colony area. Either hypothesis is supported by the many recent studies indicating that calpains are important mediators of cell-substratum attachment and cell motility. To address these issues, experiments were carried out as described under "Methods," to assess cell attachment, detachment, and mobility. Induction of calpastatin expression had no influence on attachment of trypsinized cells to culture dishes: clone 83 cells adhered to the surface within 30 min of plating and began to spread at 60 min, and ~80% of cells were spread at 120 min, independent of calpastatin expression (data not shown). There was no apparent effect of calpastatin overexpression on cell detachment produced by washing with PBS (Fig. 4*A*) or agitation (Fig. 4*B*). Moreover, cell motility did not appear to be altered: Calpastatin overexpression had no apparent effect on the rate of migration of cells into scratch-denuded areas of colonies (Fig. 5). Because attachment factors may influence calpain effects on cell migration (7), growth on culture dishes coated with 10 μg/ml fibronectin was studied. Under these conditions, ponasterone was still capable of decreasing clonal growth of clone 83 cells (data not shown).

These experiments indicated that decreased clonal growth was not the result of an obvious defect in cell-substrate attachment or motility. Because the results of these studies brought to question the importance of attachment, the growth of calpastatin-overexpressing cells in suspension culture was investigated. The growth rate of clone 83 or clone 106 cells suspended in 0.75% agar was not significantly reduced by induction of calpastatin expression (Fig. 6). Thus, attachment of cells to substratum is required for the signaling events that lead to decreased colony growth upon calpastatin overexpression.

Differential Display Analysis of Gene Expression in Calpastatin-overexpressing Cells—As discussed above, initial characterization of the cell biological properties of the calpastatin-overexpressing cells did not provide a mechanism for the decreased clonal growth observed. Moreover, in other studies it was not possible to detect differences in response to various growth factors or to identify a factor in clone 83-conditioned medium, which could account for the alteration of growth rate (data not shown). As another approach to understanding the mechanism for calpastatin's effect on growth, an evaluation of alteration of gene expression was initiated using the differential display assay, as described under "Methods."

Analysis of differential display gels utilizing 90 primer sets revealed a number of bands that differed in expression in the two different cell lines or were altered in both cell lines upon exposure to ponasterone A (not shown). Relatively few signals were specifically altered only in clone 83 cells exposed to ponasterone A, and of these only one was confirmed by Northern blot analysis (Fig. 7). A single mRNA species at about 1 kb was detected. This differential display band was re-amplified by PCR and cloned into pT-Adv vector by T/A cloning as described under "Methods." Northern blot analysis, using the insert from each of two clones as the probe, confirmed decreased expression specifically in ponasterone A-treated clone 83 cells (data not shown). The two independent clones contained the same 526-bp insert sequence, including a single coding sequence for a puta-

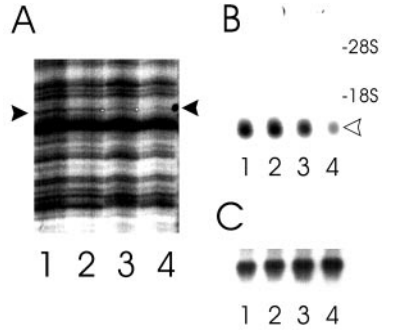

FIG. 7. **Differential display analysis identified a signal that was down-regulated only in clone 83 cells grown in medium containing ponasterone A.** *A*, differential display autoradiogram using primer set T5:P5 from the Delta differential display kit. *Lanes 1* and *2*, cDNA from mock transfected cells; *lanes 3* and *4*, cDNA from clone 83 cells. *Lanes 2* and *4* were derived from cells treated with ponasterone. The *arrowheads* show the location of a signal that was attenuated specifically in clone 83 cells grown in ponasterone-containing medium. *B*, Northern analysis of mRNAs probed with radiolabeled band excised from the differential display gel depicted in *panel A*. *Lanes* are the same as for *panel A*. *C*, re-probe of the blot in *panel B*, using β-actin cDNA as the probe.

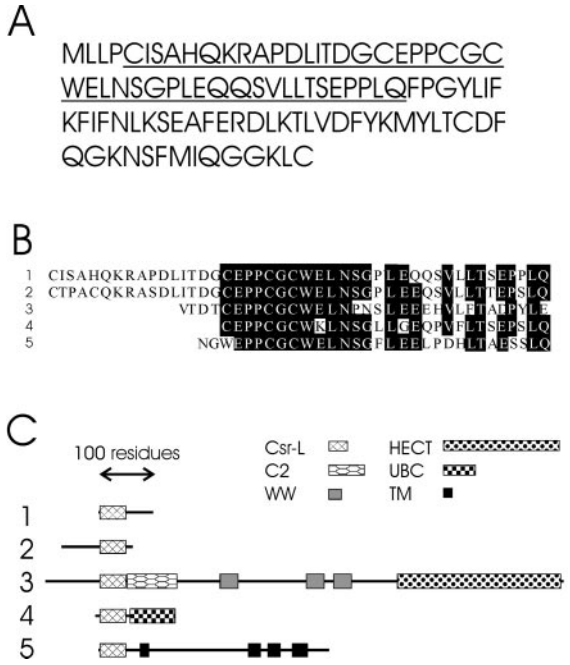

FIG. 8. **Sequence of Csr and related proteins.** *Panel A* shows the amino acid sequence of Csr, predicted from the single coding sequence in the differential display product specifically reduced upon calpastatin overexpression (GenBank™ AF484417). The *underlined portion* is the region that displays homology to other proteins. *B*, alignment of sequences with significant homology to Csr. *Line 1*, Csr. *Line 2*, mouse unknown protein (BAB31198), Blast E value = 9e-16. *Line 3*, mouse NEDD-4 ubiquitin ligase (P46935), E = 4e-07. *Line 4*, MHR6BN mouse ubiquitin conjugating enzyme (AAG27697), E = 1e-06. *Line 5*, mouse non-selective cation channel 1 (BAA25007), E = 4e-04. *C*, domains present in proteins homologous to Csr. *Numbering* is the same as for *panel B*. *Csr-L*, Csr-like region (*underlined* in *panel A*). *C2*, C2 Ca^{2+}-binding domain (pfam00168). *WW*, WW domain (pfam00397). *HECT*, HECT E3 ubiquitin-transferase domain (pfam00632). *UBC*, E2 ubiquitin-transferase domain (pfam00179). *TM*, proposed transmembrane domain.

tive 100-residue protein, which we have named Csr for calpastatin repressed (GenBank™ accession AF484417).

Blastp and Blastx analysis (26) revealed the presence of a region in Csr that appeared in several other proteins (Fig. 8). The greatest homology resided in a small mouse protein of unknown function (Fig. 8, *B* and *C*, *line 2*). Interestingly, the next closest homology was found in two apparent ubiquitin transferase enzymes, NEDD-4 E3 ubiquitin transferase, and a putative small E2 ubiquitin transferase. The other protein displaying significant homology was a putative nonspecific cation channel (27).

Decreased Clonal Growth of Capn4$^{(-/-)}$ Mouse Fibroblasts—While our studies were ongoing, Arthur *et al.* (18) disrupted the gene for the small subunit common to both m- and μ-calpain (*Capn4*) in mice, by homologous recombination techniques. The homozygous knockout mice died *in utero* (18); however, fibroblasts could be rescued from embryos, for cell culture studies. *Capn4*$^{(-/-)}$ cells did not appear to possess active conventional calpains (18) and would be predicted to display a growth phenotype similar to the calpastatin overexpressing cells reported in this contribution. In fact, colony growth was slower for *Capn4*$^{(-/-)}$ fibroblasts, but at higher plating densities there was no detectable difference in growth rate compared with *Capn4*(+/+) fibroblasts (Fig. 9).

DISCUSSION

The calpastatin-overexpressing clones established in these studies should be a valuable tool for assessing the intracellular functions of the conventional calpains, m- and μ-calpain. Unfortunately, little is known about sensitivity of the other calpain family members to inhibition by calpastatin, because they are poorly characterized as proteins or enzymes. An eye lens-specific splice variant of Capn3 does not appear to be sensitive to calpastatin (28). Importantly, calpastatin has never been shown to possess significant inhibitory potential against other important intracellular proteases, including caspases and the proteasome. The availability of these cell lines should complement studies carried out with cells derived from knockout genetics. In the latter, one can always posit alterations in developmental pathways secondary to gene ablation to account for phenotypes, or lack of phenotype, associated with the specific knockout. On the other hand, use of the *Capn4*$^{(-/-)}$ fibroblasts (Fig. 9) provides compelling evidence that it is calpastatin's ability to inhibit calpains, not some unrelated property of the inhibitor, that is important for its influence on clonal growth in our studies.

The mechanism for calpain effects on cell proliferation in colonies has not been resolved by the studies so far. However, some important aspects of this phenomenon may prove useful in further investigations. The colony phenotype, with few cells interspersed between colonies, suggested that the defect of clonal growth secondary to calpastatin expression might be the result of ablation of established calpain effects on cell detachment, attachment, or motility. Cells growing in crowded colonies might require the ability to migrate to open areas of the culture vessel to grow at optimum rates. Cells plated at higher density would not reach conditions of "crowding" prior to becoming a confluent monolayer. Attachment to a solid support appeared to be important for the growth inhibitory properties of calpastatin expression, because clone 83 cells grown in suspension were not inhibited by induction of calpastatin (Fig. 6).

Notwithstanding these considerations, no evidence for a defect in cell detachment, attachment, or motility was uncovered in the present studies. It may be that a more sophisticated analysis will reveal some degree of alteration in one or more of these properties. However, a dramatic change in cell-matrix interactions or motility seems an unlikely explanation in view of the results of our initial studies. Moreover, protein immunoblot analysis indicated that steady-state levels of spectrin, talin, and ezrin, proteins thought to be important targets for calpain effects on cytoskeletal-plasmalemma interactions during migration (11), were not detectably altered by calpastatin overexpression (data not shown).

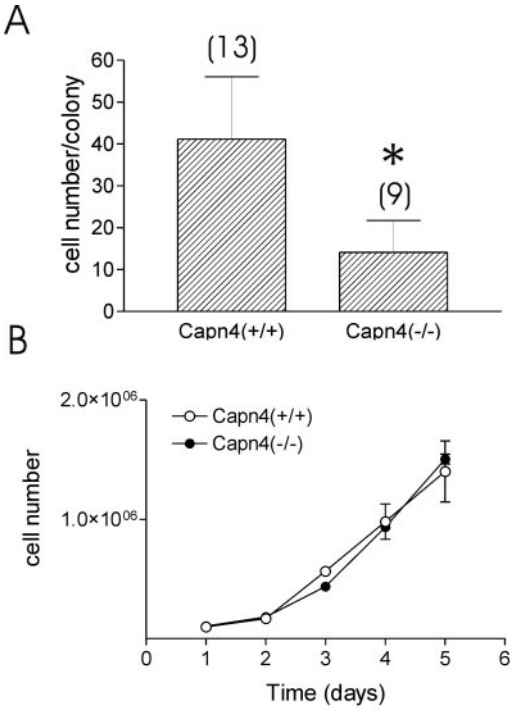

FIG. 9. **Decreased growth of *Capn4*$^{(-/-)}$ mouse fibroblast colonies.** *Capn4*$^{(-/-)}$ and *Capn4*$^{(+/+)}$ mouse fibroblasts were cultured at low density, and after 5 days cell numbers were counted for colonies randomly selected from each sample (*A*). *Asterisk*, $p < 0.0001$ compared with Capn4$^{(+/+)}$; *numbers in parentheses*, colonies counted. *B*, cells were plated at high density (10^4/cm^2), and cell numbers were determined by Coulter cell counting of trypsinized cultures.

Assessment of alterations in gene expression specifically associated with calpastatin-induced decrease of clonal growth rates might provide evidence for the molecular interactions or signaling pathways involved in this phenomenon. To initiate these efforts, differential display analysis was employed. After performing differential display reactions with 90 different primer sets, ~3000 separate PCR products were detected on gel autoradiograms. Of these only one (Csr) was found to represent an mRNA specifically down-regulated in clone 83 cells following calpastatin induction. It is intriguing that a Csr-like sequence is found in two ubiquitin transferases. However, the small Csr protein does not contain a consensus ubiquitin transferase domain. Therefore, it is premature to suggest that down-regulation of the calpain system by overexpression of calpastatin may result in decreased ubiquitin-mediated proteolysis. However, future studies may reveal such a relationship. For example, Csr might bind to a ubiquitin-conjugating enzyme and regulate its activity.

It is possible that additional genes are specifically induced or repressed following overexpression of calpastatin. However, it does not appear that the number will be great, given that one positive clone was isolated so far from ~3000 differential display bands. Our experiments have an internal positive control: Differential display signals corresponding to human calpastatin mRNA should be detectable in ponasterone-induced clone 83 cell samples. Analysis will continue, utilizing additional differential display primer pairs, until several positive control signals have been obtained. Identification of the panel of genes underexpressed or overexpressed in response to increased calpastatin will allow further studies to determine if experimental alteration of their expression levels will restore wild-type colony growth to *Capn4*$^{(-/-)}$ fibroblasts, or upon calpastatin induction in clone 83.

Lastly, it should be emphasized that identification of an effect of calpain on clonal growth has important implications for the potential therapeutic use of calpain inhibition in treatment of cancer. Calpains are currently thought to be important in muscle wasting (cachexia) in cancer (reviewed in Ref. 29). Several recent studies indicate that calpains are also involved in cell motility (7, 12, 30) and, therefore, are potential targets for novel anti-metastatic agents. If they are also important for colony growth, as the present results indicate, calpain-selective inhibitors may prove effective not only in suppressing migration of cancer cells but also in slowing clonal expansion of metastases.

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