The Major Calpain Isozymes Are Long-lived Proteins

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

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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 either major isozyme (25). A mouse monoclonal antibody developed against human *m*-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 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 *m*Ci [^{35}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 [^{35}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 *m*M leupeptin, and 10 *m*M 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 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 *m*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 *m*l of protein A/G-agarose containing bound antibodies against calpain small subunit, *m*-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 ^{35}S-labeled protein bands. A standard curve for estimating relative amounts of radiolabeled calpain in autoradiograms was prepared as follows. Different amounts of ^{35}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 ^{35}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 [^{35}S]Met from *m*- 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 *m*M phosphorothioate ODNs as a complex with 5 *m*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 *m*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 × *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 [^{35}S]Met and chased with nonradioactive Met as described under "Experimental Procedures." Control experiments showed that the antibodies against m-calpain and *m*-calpain large subunits, and the small subunit which is present in both calpain isozymes, quantitatively im-

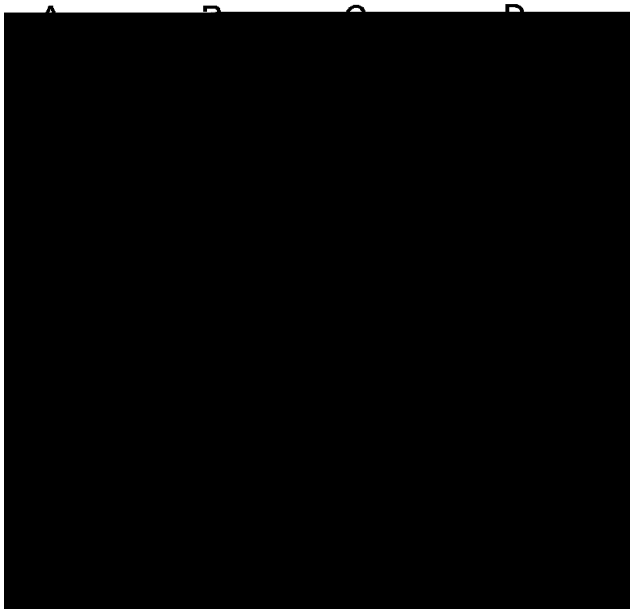

FIG. 1. **Immunoprecipitation of calpains from WI-38 fibroblasts and calpastatin from C-33A cells.** Calpains or calpastatin were immunoprecipitated from ^{35}

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}S-labeled calpains per 48 h (Table II). TE2 cells and C-33A cervical carcinoma cells were also analyzed for loss of ^{35}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 oligodeoxyribonucleotide 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

to 0.2 *m*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

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

REFERENCES

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