Isolation of a Chinese Hamster Ovary Cell Clone Possessing Decreased *m*-Calpain Content and a Reduced Proliferative Growth Rate*

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A Chinese hamster ovary cell line (CHO

from Hyclone. Cell culture grade dimethyl sulfoxide (catalog D5879), MTT, DEAE-Sepharose, and vitamin-free casein were obtained from Sigma. ZLLY-CHN_2 was synthesized from the peptidyl acid anhydride and ethereal diazomethane (25). It was added to culture medium from a 1000-fold concentrated stock solution in dimethyl sulfoxide. Bovine myocardial m-calpain (15) and human erythrocyte calpastatin (26) were prepared as described previously. Casein to be used as a calpain substrate was labeled with [^{14}C]formaldehyde by reductive methylation (27). A polyclonal antibody developed against rat m-calpain, which cross-reacts with hamster *m*- and m-calpains, was a generous gift from Dr. John Elce, Queen's University, Kingston, Ontario, Canada.

Mammalian Cell Culture—Chinese hamster ovary dhfr$^-$ cells stably transfected with pSV2dhfr plasmid (28) were a kind gift from Dr. Edwin Sanchez, Dept. of Pharmacology. They will be hereafter called CHO^p cells expressly for ease of discussion. Cells were cultured at 37 °C in a 5% CO_2 atmosphere. Unless otherwise indicated, the medium utilized was IMDM supplemented with L-glutamine, 25 mM Hepes, and 10% bovine calf serum.

Isolation and Characterization of a ZLLY-CHN_2-resistant CHO Clone—To minimize the potential for selection of clones overexpressing *mdr-1* gene, which can catalyze efflux of small peptides (29), we treated 50–70% confluent CHO^p cells for brief periods (16 h) with 70 *m*M ZLLY-CHN_2. Cells were then allowed to recover in medium without inhibitor. Thus, continuous exposure to ZLLY-CHN_2, which might select for cells capable of efficiently exporting the inhibitor, was avoided. This concentration of inhibitor was toxic to a large fraction of CHO^p cells, and recovery of survivors occurred over a period of 6–8 days of culture in the absence of inhibitor. After two treatments as described above, the CHO^p cells were plated in 2-cm^2 culture plate wells at serial dilutions of 30–1000 cells/well and treated once more with 70 *m*M ZLLY-CHN_2 for 16 h. The SHI cells used in this study appeared as a single colony in one of the wells after 1 week of recovery from this final treatment. They were allowed to grow for another week to ascertain that no other colonies appeared in the well and to allow accumulation of sufficient cells to transfer to a 25-cm^2 flask for further growth.

Cell Growth Assays—Proliferative cell growth was measured directly by counting suspended trypsinized cells using a hemocytometer. At least six separate counts were taken for each sample, and the average was taken for calculating cell number. In some experiments, cell growth was estimated by measurement of mitochondrial MTT reductase activity as described previously (30), with minor modifications. Cells were grown in 24-well plates in 1 ml of medium. MTT was added to a final concentration of 2 mM, and the cells were incubated for 30 min at 37 °C. Culture medium was removed, and 800 *m*l of 95% ethanol was added to the wells to solubilize the blue formazan product of MTT reduction. The ethanol suspension was centrifuged, and the A_{590} of the supernatant was recorded.

Cell Survival Assay—CHO^p or SHI cells were obtained by mitotic shake off from 70% confluent 75-cm^2 flasks. Approximately 1000 cells/well were plated in 2.5 ml of IMDM containing 10% bovine calf serum in 9.5-cm^2 wells. After 1 h, medium was changed to remove cells that did not attach. Control experiments indicated that plating efficiency under these conditions was approximately 50% for either CHO^p or SHI cells. ZLLY-CHN_2 was added to a final concentration of 70 *m*M, and cells were exposed to this inhibitor for 16 h, washed twice with phosphate-buffered saline, and allowed to recover in medium minus ZLLY-CHN_2. After 12 days of recovery, cells were stained for 30 min with 2 mM MTT in culture medium, and colonies $\geq$16 cells were counted.

To investigate the effect of increased *m*-calpain content on cell survival, mitotic SHI cells were electroporated in the presence of *m*-calpain as described below, and approximately 1000 viable cells (determined by trypan blue dye exclusion) were plated per well in 9.5-cm^2 culture plate wells. After 24 h to allow recovery from electroporation, the cells were exposed to 70 *mM ZLLY-CHN_2* for 16 h and analyzed as described above for colony formation.

Tritiated Thymidine Incorporation—Mitotic CHO^p or SHI cells were collected by shake off, and approximately 10,000 cells were plated per well in 48-well plates. At various times, 0.5 *m*Ci of [^{3}H]thymidine was added to the culture medium in individual wells. After 30 min of incubation, medium was removed, and thymidine incorporation was assayed as described previously (31). Briefly, the cells were extracted with buffer containing SDS, DNA was precipitated with trichloroacetic acid, and the NaOH-solubilized DNA pellet was assayed for ^{3}H by liquid scintillation counting.

Mitotic Index—Cells were harvested by trypsinization and fixed with methanol:acetic acid (3:1). Mitotic indices were measured by light microscopy on a minimum of 1000 Giemsa-stained cells.

Preparation of Cell Homogenate Supernatants—Unless otherwise indicated, cells were grown in 15 ml of IMDM in 75-cm^2 flasks until confluent. The cells were washed two times with 5 ml of Hanks' balanced salt solution at 37 °C and scraped in 0.5 ml of ice-cold 50 mM Mops, 5 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, pH 7.0. The

munoreactive bands were stained with indolyl phosphate and nitro blue tetrazolium (37).

Electroporation—SHI cells were collected by shake off from 12 70% confluent T-75 culture flasks, washed by centrifugation several times with sterile phosphate-buffered saline, and subjected to electroporation based on a previously reported protocol (38). Two aliquots containing 1.25 3 10^6 cells each were suspended in 50 *m*l of phosphate-buffered saline containing 0.5 mM EGTA, 0.1 mM dithiothreitol, and 2 mg of either BSA or human erythrocyte *m*-calpain/ml. The samples were incubated on ice in electroporation cuvettes and then electroporated in a Bio-Rad Gene Pulser electroporator at 0.26 kV using a pulse time of 20–26 s at a capacitance of 0.125 microfarads. The cells were immediately diluted with 1 ml of warm IMDM containing 0.1 mg of human erythrocyte calpastatin to inhibit extracellular calpain and gently suspended.

For measuring uptake of calpain by electroporation, the cells were treated with 100 *m*g of trypsin/ml for 2–3 min, harvested by centrifugation, and immediately suspended in hot SDS-sample preparation buffer. The solubilized proteins were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. The first antibody was a monoclonal antibody which is highly species-specific for human *m*-calpain large subunit, and therefore, the hamster *m*

Decreased activity of SHI *m*-calpain could have been the result of a lower content of this isozyme or some covalent post-translational modification (*e.g.* phosphorylation) which decreased its specific activity. To resolve this issue, samples of pooled *m*-calpain and m-calpain activity peaks from the experiments depicted in Fig. 5, *panels A* and *B*, were subjected to immunoblotting experiments. *Panels A* and *B*, fractions 12–15, were separately pooled for CHO^p and SHI m-calpain. For CHO^p *m*-calpain, *panel A*, fractions 8 and 9 were pooled. For SHI *m*-calpain, *panel B*, fractions 9 and 10 were pooled. As expected, CHO^p and SHI cells exhibited the same amount of m-calpain immunoreactivity; however, the pooled *m*-calpain peak from the SHI cell sample contained less immunoreactivity than the CHO^p cell sample (Fig. 5, *panel C*; *lanes 4* and *3*, respectively). Further analysis of the pooled SHI *m*-calpain peak failed to show any substantial alteration in calcium requirement ($A_{0.5}$ for Ca^{21} 5 28 *mM* for SHI calpain and 41 *mM* for CHO^p calpain) and sensitivity to inhibition by ZLLY-CHN

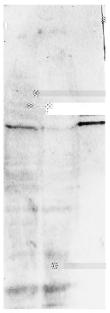

FIG. 6. **Accumulation of human *m*-calpain in electroporated SHI cells.** SHI cells were electroporated in the presence of 2 mg of human *m*-calpain/ml and then analyzed for *m*-calpain large subunit by Western blotting as described under "Experimental Procedures." A control sample of cells was treated under the same conditions but not subjected to the electroporation step. *Lane 1*, 10^6 electroporated cells; *lane 2*, 10^6 control cells; *lane 3*, 50 ng of purified human *m*-calpain.

for CHO^p calpain). These studies suggest that SHI cells contain a relatively small amount of normal *m*-calpain isozyme.

Restoration of Wild-type Growth Rate by Electroporation of m-Calpain into SHI Cells—When SHI cells were subjected to electroporation in the presence of 2 mg of human *m*-calpain/ml, as described under "Experimental Procedures," they accumulated approximately 25 ng of *m*-calpain/10^6 cells (Fig. 6). This is nearly the same as the content of *m*-calpain in wild-type CHO^p cells. The latter is estimated to be 30 ng/10^6 cells, based on the specific activity of purified *m*-calpain (5.4 units/mg protein), and the *m*-calpain activity recovered from CHO^p cells after Bio-Gel chromatography as depicted in Fig. 4 (5 units from two confluent T-75 flasks or 30 3 10^6 cells). The calpain was internalized and not simply bound to the exterior of the cells, since it was not removed by trypsin treatment (see "Experimental Procedures"). Uptake of the human *m*-calpain sensitized the SHI cells to the toxic effects of ZLLY-CHN_2 (Fig. 8); whereas electroporation in the presence of BSA produced little if any increase in ZLLY-CHN_2 toxicity (compare Fig. 8, BSA sample, with Fig. 1, SHI cells).

A 24-well plate was seeded at approximately 5000 cells/cm^2 with cells electroporated in the presence of 2 mg of human *m*-calpain or albumin/ml. Cell counts were determined at various times after electroporation. Fig. 7 presents the results of one of two experiments that produced essentially the same results. Between 2 and 24 h after electroporation, there was little or no cell growth, whether the cells were electroporated in the presence of calpain or BSA. This presumably reflects recovery of the cells following electroporation. Between 24 and 48 h after electroporation in the presence of *m*-calpain, the doubling time was 18 h, close to the wild-type growth rate. The BSA-treated cells grew with a doubling time of 29 h after the initial 24-h lag, consistent with the growth rate of untreated SHI cells. As indicated in Fig. 7, the increased cell number for the calpain-electroporated cells was statistically significant at 48 and 72 h. In an independent experiment, 72-h post-electroporation values of 317 6 8 and 199 6 64 (S.D.) cells/mm^2 were obtained for cells electroporated in the presence of *m*-calpain and BSA, respectively (n 5 3 culture wells, p 5 0.033). The increased growth rate of the calpain-treated cells was transitory. In the time interval between 48 and 120 h, the apparent population doubling time in this experiment was 34 h. This is slightly longer than for untreated SHI cells. However, in the repeat experiment, the doubling time during this interval was 30 h, which is nearly the same as for untreated SHI cells. After 48 h of culture, little human *m*-calpain was present per SHI cell (Fig. 7, *inset*). There was a faint band at this position in the protein immunoblot. However, it is not noticeable in the photograph.

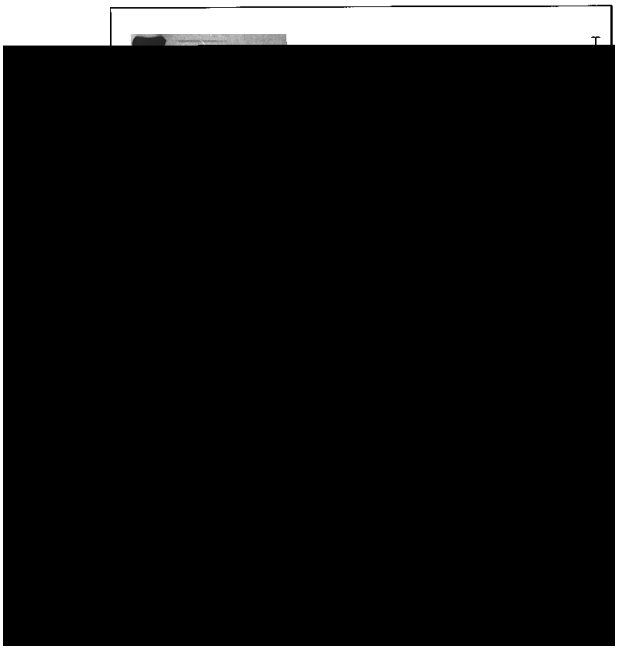

FIG. 7. **Introduction of human *m*-calpain into SHI cells increased growth rate.** SHI cells were electroporated in the presence of 2 mg of *m*-calpain/ml (*filled circles*) or 2 mg of BSA/ml (*open circles*) and then plated in wells as described under "Experimental Procedures." At various times after electroporation, cell counts were taken. *Error bars* indicate mean 6 S.D. for three wells. The absence of error bars means that they are within the area of the data points. *p* values reported in *parentheses above* data refer to comparisons of calpain and BSA cell numbers at the same time points. *Inset*, *m*-calpain-electroporated SHI cells were removed by trypsin treatment immediately after attaching to wells (0 time) and 48 h after electroporation. Cell counts were taken, and 3 3 10^5 cells were electrophoresed and analyzed by immunoblotting for human *m*-calpain. *S*, 800 ng of human *m*-calpain large subunit.

DISCUSSION

Isolation and Initial Characterization of SHI Cells—By culturing CHO^p cells in the presence of ZLLY-CHN_2, the SHI cell line was selected. SHI cells were resistant to the toxicity of this cell-permeant, irreversible calpain inhibitor (Fig. 1). In other studies, we found that the SHI cells were as sensitive as CHO^p cells to inhibition of cell growth by ZLLY-CHN_2 (not shown). Thus, it did not seem likely that SHI cells contained a form of calpain which was insensitive to this inhibitor. Subsequently, it was demonstrated that partially purified SHI *m*-calpain was approximately as sensitive as CHO^p *m*-calpain to inhibition by ZLLY-CHN_2. Moreover, the *m*-calpain present in SHI cells did not appear to differ significantly from CHO^p *m*-calpain in chromatographic properties (Figs. 4 and 5), large subunit molecular mass (Fig. 5, *panel C*), Ca^{21}-requirement, or sensitivity to inhibition by calpastatin. These results indicate that SHI cells express a form of *m*-calpain that is similar to, or identical with, wild-type *m*-calpain.

The molecular mechanism of SHI cell escape from the toxicity of ZLLY-CHN_2 remains a matter for speculation. SHI cells appeared to possess 50–70% less *m*-calpain than the parental cell line (Figs. 4 and 5). More detailed analysis is required to determine whether this defect is at the transcriptional, translational, or post-translational level. Whatever the mechanism may be, the decreased content of *m*-calpain in these cells could be related to their ability to survive treatment with ZLLY-CHN_2, since there are well-characterized examples of enhanced survival of cells which underproduce target proteins for cytotoxic agents (41). In agreement with this notion, electropo-

ration of purified *m*-calpain into SHI cells decreased their survival rate upon exposure to ZLLY-CHN_2 (Fig. 8). It should be noted that more *m*-calpain-electroporated SHI cells survived this treatment than CHO^p cells (compare Figs. 1 and 8). This may reflect the short window for ZLLY-CHN_2 treatment, 24 h after electroporation to allow recovery of SHI cell growth but before the 48 h time point, when the transitory effect of electroporated *m*-calpain on growth rate ends. This may allow some

9. Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R. E., and Cohen, L. H. (1991) *J. Biol. Chem.* **266,** 16376–16379
10. Jariel-Encontre, I., Pariat, M., Martin, F., Carillo, S., Salvat, C., and Piechaczyk, M. (1995) *J. Biol. Chem.* **270,** 11623–11627
11. Maki, M., Hatanaka, M., Takano, E., and Murachi, T. (1990) *Intracellular Calcium-dependent Proteolysis* (Mellgren, R. L., and Murachi, T., eds) pp. 37–54, CRC Press, Inc., Boca Raton, FL
12. Suzuki, K., Saido, T. C., and Hirai, S. (1992) *Ann. N. Y. Acad. Sci.* **674,** 218–227
13. Hirai, S.-I., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991) *FEBS Lett.* **287,** 57–61
14. Carillo, S., Pariat, M., Steff, A.-M., Roux, P., Etienne-Julan, M., Lorca, T., and Piechaczyk, M. (1994) *Oncogene* **9,** 1679–1689
15. Mellgren, R. L. (1991) *J. Biol. Chem.* **266,** 13920–13924
16. Mellgren, R. L., Song, K., and Mericle, M. T. (1993) *J. Biol. Chem.* **268,** 653–657
17. Takahashi, K. (1990) in *Intracellular Calcium-dependent Proteolysis* (Mellgren, R. L., and Murachi, T., eds) pp. 55–74, CRC Press, Inc., Boca Raton, FL
18. Wang, K. K. W. (1990) *Trends Pharmacol. Sci.* **11,** 139–142
19. Mehdi, S. (1991) *Trends Biochem. Sci.* **16,** 150–153
20. Crawford, C. (1990) in *Intracellular Calcium-dependent Proteolysis* (Mellgren, R. L., and Murachi, T., eds) pp. 75–89, CRC Press, Inc., Boca Raton, FL
21. Anagli, J., Hagmann, J., and Shaw, E. (1991) *Biochem. J.* **274,** 497–502
22. Anagli, J., Hagmann, J., and Shaw, E. (1993) *Biochem. J.* **289,** 93–99
23. Shaw, E., and Green, G. D. J. (1981) *Methods Enzymol.* **80,** 820–825
24. Mellgren, R. L., Shaw, E., and Mericle, M. T. (1994) *Exp. Cell Res.* **215,** 164–171
25. Crawford, C., Mason, R. W., Wikstrom, P., and Shaw, E. (1988) *Biochem. J.* **253,** 751–758
26. Mellgren, R. L. (1988) *Biochem. Biophys. Res. Commun.* **150,** 170–176
27. Tack, B. F., Dean, J., Eilat, D., Lorenz, P. E., and Schechter, A. N. (1980) *J. Biol. Chem.* **255,** 8842–8847
28. Urlaub, G., and Chasin, L. A. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 4216–4220
29. Sharma, R. C., Inoue, S., Roitelman, J., Schimke, R. T., and Simoni, R. D. (1992) *J. Biol. Chem.* **267,** 5731–5734
30. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63
31. Freshney, R. I. (1987) *Culture of Animal Cells,* 2nd Ed., pp. 236–237, Wiley-Liss, Inc., New York
32. Mellgren, R. L., Repetti, A., Muck, T. C., and Easly, J. (1982) *J. Biol. Chem.* **257,** 7203–7209
33. Barrett, A. J. (1972) *Anal. Biochem.* **47,** 280–293
34. Illingworth, B., and Cori, G. T. (1953) *Biochem. Prep.* **3,** 1–9
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
36. Laemmli, U. K. (1970) *Nature* **227,** 680–685
37. Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem.* **136,** 175–179
38. Lukas, J., Bartek, J., and Strauss, M. (1994) *J. Immunol. Methods* **170,** 255–259
39. Katunuma, N., and Kominami, E. (1983) *Curr. Top. Cell. Regul.* **22,** 71–101
40. Goll, D. E., Edmunds, T., Kleese, W. C., Sathe, S. K., and Shannon, J. D. (1985) *Prog. Clin. Biol. Res.* **180,** 151–164
41. Katzung, B. G. (1992) *Basic and Clinical Pharmacology,* 5th Ed., pp. 623–624, 776, Appleton & Lange, Norwalk, CT
42. Schollmeyer, J. E. (1988) *Science* **240,** 911–913
43. March, K. L., Wilensky, R. L., Roeske, R. W., and Hathaway, D. R. (1993) *Circ. Res.* **72,** 413–423