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

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A Chinese hamster ovary cell line (CHO^P) was cultured in the presence of benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone (ZLLY-CHN_2), to select for resistance to this cell-permeant calpain inhibitor. A clone isolated after several courses of exposure (SHI cells) demonstrated decreased sensitivity to ZLLY-CHN_2 toxicity and a decreased growth rate. SHI cells also possessed less μ-calpain isozyme relative to CHO^P cells, as determined by activity measurement or by protein immunoblotting. Activities of m-calpain, calpastatin, cathepsin B, cathepsin L, and glycogen phosphorylase were not altered. SHI μ-calpain was partially purified by sequential chromatography on Bio-Gel A-1.5m and DEAE-Sepharose. Its chromatographic behavior in either system was the same as for CHO^P μ-calpain. Further studies with the partially purified SHI and CHO^P μ-calpain fractions failed to distinguish any difference in Ca^{2+} requirement or in sensitivity to inhibition by calpastatin or ZLLY-CHN_2 for these enzymes. These experiments suggest that SHI cells underproduce a form of μ-calpain which is very similar to, if not identical with, CHO^P μ-calpain. SHI cells displayed a population doubling time of 29 h compared with 19 h for CHO^P cells. The decreased growth rate of SHI cells was the result of a prolonged G_1 phase. Introduction of purified human μ-calpain into SHI cells by electroporation transiently restored the growth rate and also increased toxicity associated with exposure to ZLLY-CHN_2. SHI cells should be a valuable model in further studies to delineate the function of μ-calpain in cell proliferative growth.

Proliferation of cells in multicellular organisms is a highly regulated process involving complex extracellular and intracellular signaling events that must occur under strict spatial and temporal coordination (1, 2). Recently, it has become apparent that controlled proteolysis of specific growth regulating proteins is an essential feature of normal cell division (3–6) and may contribute to pathologic cell proliferation as well (7).

Thus far, there is substantial evidence only for participation of the ATP and ubiquitin-dependent proteolytic system in proteolysis of growth associated proteins (7–9). However, it is likely that other regulated intracellular proteolytic enzymes are necessary for cell proliferation. For example, it has recently been shown that conjugation with ubiquitin is not necessary for the degradation of the AP-1 complex constituent, c-Jun (10). An intracellular proteolytic system that could function in cell growth comprises two calcium-dependent proteases, m-calpain (calpain II, m-CANP) and μ-calpain (calpain I, μ-CANP), and their specific inhibitor protein, calpastatin (11, 12). The calpains have an absolute requirement for Ca^{2+}. Given the important role of intracellular Ca^{2+} as a second messenger, it seems likely that calpain-catalyzed proteolysis is required for some Ca^{2+}-responsive intracellular processes, including cell proliferation. c-Jun and other growth-related transcription factors are excellent calpain substrates (13, 14). Transfection of NIH3T3 or F9 cells with calpastatin sense RNA appeared to increase c-Jun stability (13). Calpastatin antisense RNA had the opposite effect. Moreover, DNA-binding proteins in isolated rat liver nuclei required very low Ca^{2+} concentrations for proteolysis by calpains (15, 16). Thus c-Jun or other transcription factors bound to DNA are potential substrates for calpains at the low concentrations of intracellular Ca^{2+} generated during Ca^{2+}-dependent signal transduction events.

Unfortunately, it has proven difficult to gain insight into the physiologic function of the calpains; they display a great deal of flexibility in substrate specificity (17), and most importantly, a calpain-specific, cell-permeant inhibitor has not yet been developed, although much effort has been invested in this direction (18–20). The best specificity appears to have been achieved with a peptidyldiazomethyl ketone, ZLLY-CHN_2,[1] which inhibits calpains and cathepsin L (21). Treatment of platelets with radioiodine-labeled ZLLY-CHN_2 and subsequent addition of Ca^{2+} ionophore resulted in specific radiolabeling of calpain large subunit (21, 22). The diazomethyl ketones are irreversible, mechanism-based inhibitors (20, 23) and allow one to inhibit most of the calpain activity in platelets (21, 22) or cultured cells (24). We have recently utilized ZLLY-CHN_2 to show that calpain activity is essential for growth of various cultured animal cells (24). In the present study, this inhibitor was used as a selecting agent to isolate a CHO cell clone which has diminished μ-calpain content and a reduced proliferative growth rate.

EXPERIMENTAL PROCEDURES

Materials—Electrophoresis and protein immunoblotting supplies, as well as Bio-Gel A-1.5m, were obtained from Bio-Rad. IMDM was purchased from Life Technologies, Inc., and bovine calf serum was obtained

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[1] The abbreviations used are: ZLLY-CHN_2, benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone; IMDM, Iscove's modified Dulbecco's medium; Me_2SO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Mops, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; $A_{0.5}$, concentration required to produce half-maximum activity; $I_{0.5}$, concentration required to produce half-maximum inhibition.

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 μ- 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 μM 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 μM 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 μl 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 μM, 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 ≥16 cells were counted.

To investigate the effect of increased μ-calpain content on cell survival, mitotic SHI cells were electroporated in the presence of μ-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 μM *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 μCi 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 cells were then homogenized in a small frosted-glass homogenizer. Cell homogenates were centrifuged for 10 min at 12,000 × *g* to produce a low speed supernatant fraction.

Bio-Gel A-1.5m Chromatography—Generally cell supernatants from three 75-cm^2 flasks were pooled for each Bio-Gel chromatography experiment. A 1-ml sample of supernatant, equivalent to approximately ⅔ of the total, was applied at 7 °C to a 1 × 28-cm column equilibrated in 50 mM imidazole-HCl, 0.2 mM EGTA, 100 mM NaCl, 1 mM dithiothreitol, pH 7.8. Fractions of 0.5 ml were collected.

DEAE-Sepharose Chromatography—Total calpain activity fractions from Bio-Gel chromatography runs were pooled, avoiding fractions which contained measurable calpastatin activity, and diluted with Bio-Gel equilibration buffer minus NaCl to a final NaCl concentration of 70 mM. Pooled calpain fractions were applied at 7 °C to a 4-ml DEAE-Sepharose column equilibrated in 50 mM imidazole-HCl, 0.2 mM EGTA, 1 mM dithiothreitol, 50 mM NaCl, pH 7.8. Calpain activities were eluted with a 20-ml linear concentration gradient of NaCl, 50–500 mM, in equilibration buffer. One-ml fractions were collected.

Calpain and Calpastatin Assays—Calpain activity was determined by release of trichloroacetic acid-soluble fragments from [^{14}C]methyl casein essentially as described previously (32). Ten microliters of the calpain sample was assayed for 20–30 min. Total calpain activity was determined by assaying in the presence of 5 mM Ca^{2+}, and the μ-calpain isozyme was selectively assayed by using 0.2 mM Ca^{2+} in the assay. m-Calpain activity was calculated by subtracting μ-calpain activity from total calpain activity. To correct for background proteolysis in cell extracts or chromatography fractions, blanks contained all of the components of the assay mixture including the cell homogenates or column fractions, as well as 16 μg of human calpastatin/ml. The latter is a highly specific inhibitor of calpains (20). Background proteolysis accounted for no more than 2% of the total caseinolytic activity in Bio-Gel chromatography fractions and was not detectable in DEAE-Sepharose column fractions. A unit of calpain activity produces 1 μg of trichloroacetic acid-soluble fragments of casein per min under standard assay conditions.

Calpastatin activity was determined by its ability to inhibit a defined amount of purified bovine myocardial m-calpain in the standard ^{14}C-caseinolytic assay. Column fractions were heated at 100 °C for 10 min and then centrifuged at 12,000 × *g* for 10 min. Calpastatin is a heat-stable protein and remains in the supernatant fraction. Ten-μl samples of the supernatant were assayed for inhibitory activity utilizing 0.4 unit of purified m-calpain in the incubation mixture. A unit of calpastatin produces 50% inhibition of 1 unit of calpain in the standard calpain assay.

Other Enzyme Assays—Cathepsin L-like activity in cell homogenate supernatants was determined by a modification of the caseinolytic calpain assay. The pH of the buffer was adjusted to 6.0; 10 μM pepstatin A was added to inhibit cathepsin D-like activity, and Ca^{2+} was replaced by 0.5 mM EDTA. Ten microliters of supernatant was added to 40 μl of substrate at 37 °C. After 10–16 h, trichloroacetic acid was added to 2.5%, and trichloroacetic acid-soluble radioactivity was measured as described previously (32). Other samples of supernatant were assayed in the presence of ZLLY-CHN_2, and cathepsin L-like activity was taken as the difference in radioactivity between the assays with and without this inhibitor. One unit of cathepsin L-like activity produces 1 μg of trichloroacetic acid-soluble casein per h. Cathepsin B-like activity was assayed using benzoylarginine β-naphthylamide as substrate (33). A unit produces 1 nmol of β-naphthylamine per min under the standard assay conditions. Total glycogen phosphorylase was measured as described previously (34) in the presence of mM AMP. A unit of phosphorylase produces 1 nmol of inorganic phosphate per min.

Protein Determination—The protein concentration of cell homogenate supernatants and column fractions was determined by the method of Lowry *et al.* (35) using BSA as the standard.

Protein Immunoblotting—Proteins and cell homogenates were heated to 100 °C in the presence of SDS and mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis on 10% slab gels using the Laemmli buffer system (36). CHO^p cell or SHI cell proteins in the gels were transferred to nitrocellulose sheets and immunostained for m-calpain and μ-calpain large subunits utilizing the anti-rat calpain immune serum obtained from Dr. Elce as first antibody and alkaline phosphatase-conjugated second antibody. Human calpain electroporated into SHI cells was detected with a monoclonal antibody derived using human erythrocyte μ-calpain large subunit as the antigen. Im-

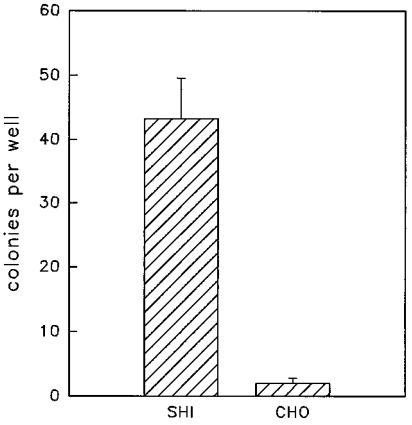

FIG. 1. **Toxic effect of ZLLY-CHN$_2$ on CHO^P and SHI cells.** Toxicity resulting from a single 16-h exposure to 70 μM ZLLY-CHN$_2$ was assessed by a cell survival assay as described under "Experimental Procedures." *Error bars*, standard deviation (n = 4 wells).

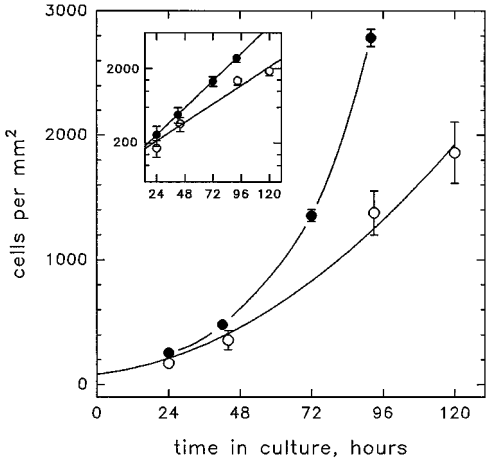

FIG. 2. **Growth curves for CHO^P and SHI cells.** Cells were plated at 100 cells/mm^2 in 24-well plates. At various times triplicate samples were trypsinized for cell counts. *Filled circles*, CHO^P cells; *open circles*, SHI cells. Replot of the data on a semi-logarithmic scale (*inset*) showed population doubling times of 19 h for CHO^P cells and 29 h for SHI cells. *Error bars* indicate standard deviation.

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 1.25×10^6 cells each were suspended in 50 μl of phosphate-buffered saline containing 0.5 mM EGTA, 0.1 mM dithiothreitol, and 2 mg of either BSA or human erythrocyte μ-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 μg 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 μ-calpain large subunit, and therefore, the hamster μ-calpain in control SHI cells was not detected. Approximation of the amount of μ-calpain incorporated into SHI cells was based on visual comparison of immunostaining of SHI cell extracts with immunostaining of known amounts of purified human μ-calpain, electroblotted from the same gel slab.

In other experiments, the electroporated cells were diluted 20-fold in IMDM medium and plated in 24-well culture plates at approximately 5,000 cells/well. Following a 2-h incubation to allow cell attachment, medium was replaced. At various times after plating, cell counts were taken to measure proliferative growth. In a control experiment, it was found that addition of calpain and calpastatin to cells under the same conditions as described above, but without electroporation, did not influence cell growth.

Statistical Analysis—p values, where indicated, were derived from Student's t test for nonpaired (independent) samples.

RESULTS

Growth Characteristics of SHI Cells—As described under "Experimental Procedures," SHI cells were selected from the parental CHO^P cell line on the basis of their resistance to ZLLY-CHN$_2$ toxicity. Resistance of the isolated SHI cells was confirmed by a survival assay performed as described under "Experimental Procedures" (Fig. 1).

Initial characterization studies were performed to compare growth properties of SHI cells compared with the parental CHO^P cell line. SHI cells grew at a substantially lower rate than CHO^P cells, assayed either by MTT reductase activity (not shown) or direct cell counting (Fig. 2). Under standard culture conditions, the SHI cells had a population doubling time of 29 h, compared with 19 h for CHO^P cells. Studies of tritiated thymidine incorporation in CHO^P and SHI cells synchronized in mitosis indicate that the major difference in growth rate occurred between mitosis and S phase. This interval was prolonged by 8–10 h in SHI cells (Fig. 3). The decreased growth rate could have been the result of either decreased rate of exit from mitosis or an increased G_1 to S phase interval. However, the mitotic index of the SHI cells was 3.4 ± 0.3% compared with 6.7 ± 1.1% for CHO^P cells (n = 4), indicating that mitosis is not prolonged in the former. Therefore, the SHI cells appear to take longer than CHO^P cells to progress through G_1 phase. SHI cells have maintained this reduced growth rate after more than 20 passages in IMDM containing 10% bovine calf serum.

Calpain and Calpastatin Content of SHI Cells—Supernatants were prepared from CHO^P or SHI cell homogenates as described under "Experimental Procedures." Samples were applied to a gel filtration column, and fractions were assayed for calpains and calpastatin. Several independent experiments indicated that CHO^P cell μ-calpain represented 7–10% of total calpain (Fig. 4, *panel A*). SHI cells contained significantly less μ-calpain activity (Fig. 4, *panel B*). Calpastatin content was essentially the same for both cell lines.

Cathepsin L-like Activity in SHI Cells—In addition to the calpains, the other major intracellular target of ZLLY-CHN$_2$ appears to be cathepsin L (21, 22). To determine whether SHI cells had altered cathepsin L content, cell homogenate supernatants from SHI and CHO^P cells were assayed for cathepsin L-like activity as described under "Experimental Procedures." Casein was chosen as the substrate for these experiments because cathepsin L has more activity on protein substrates than other intracellular cysteine proteases, including cathepsins B and H (39). Cathepsin L-like activity was modestly increased in SHI cells at best (Table I). Moreover, assays for cathepsin B, a protease which is not very sensitive to inhibition by ZLLY-CHN$_2$ (25), also revealed a slight increase, as did glycogen phosphorylase activity. These data do not support the notion that ZLLY-CHN$_2$ treatment selected for a clone possessing altered cathepsin L levels.

Characterization of SHI μ-Calpain—To further explore possible differences between μ-calpain activity present in SHI and CHO^P cells, pooled Bio-Gel fractions from chromatography runs of SHI or CHO^P cell extracts were subjected to DEAE-Sepharose chromatography. SHI and CHO^P cell μ-calpain activities eluted at the same NaCl concentration, 0.15 M (Fig. 5, *panels A* and *B*), which is characteristic for μ-calpain from several different species (40).

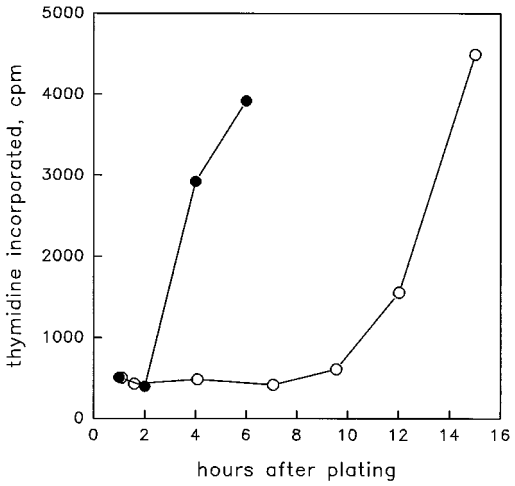

FIG. 3. **Delayed entry of mitotic SHI cells into S phase.** SHI cells or CHO^P cells were collected by mitotic shake off and plated at a density of 10^4 cells/cm^2 in 0.5-cm^2 wells. At various times, DNA synthesis was assayed by [^{3}H]thymidine incorporation as described under "Experimental Procedures." *Filled circles*, wild-type CHO^P cells; *open circles*, SHI cells.

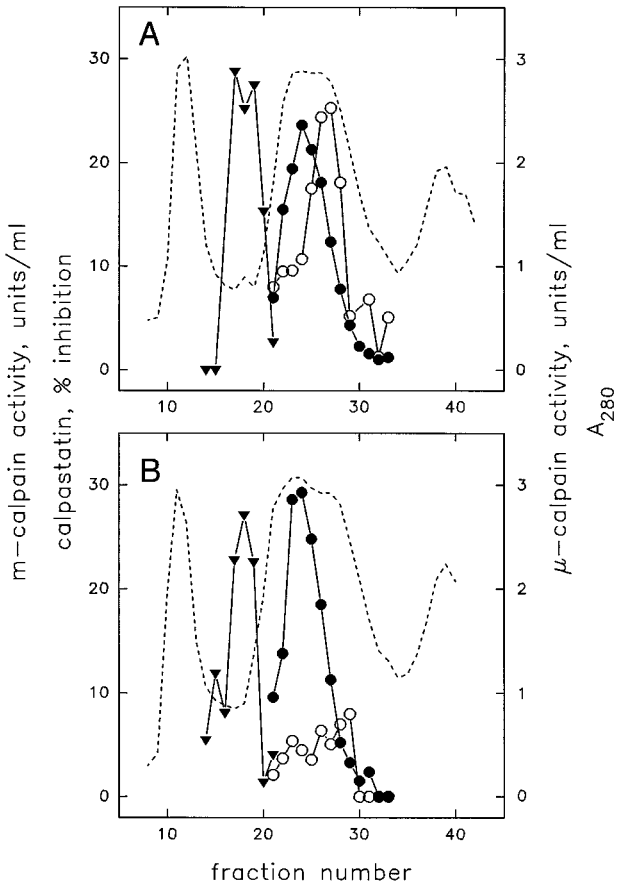

FIG. 4. **Bio-Gel A-1.5m chromatography of cell homogenate supernatants.** Supernatants derived from cell homogenates of CHO^P cells (*panel A*) or SHI cells (*panel B*) were subjected to Bio-Gel chromatography as described under "Experimental Procedures." *Filled circles*, m-calpain activity; *open circles*, μ-calpain activity; *triangles*, calpastatin activity; *dashed lines*, A_{280}. Protein loads on the chromatography runs were 3 mg.

Decreased activity of SHI μ-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

TABLE I
Specific activities of cathepsin L- and B-like activities and glycogen phosphorylase in CHO^P and SHI cell extracts

Enzymatic activity	Specific activity	
	CHO	SHI
	units/mg protein[a]	
Cathepsin L-like	0.797 ± 0.101	0.844 ± 0.111
Cathepsin B-like	0.520 ± 0.027	0.699 ± 0.048
Glycogen phosphorylase	48.9 ± 4.5	62.5 ± 5.6

[a] Units for each enzymatic activity are defined in the "Materials and Methods." Values are means ± S.D. for three culture dishes.

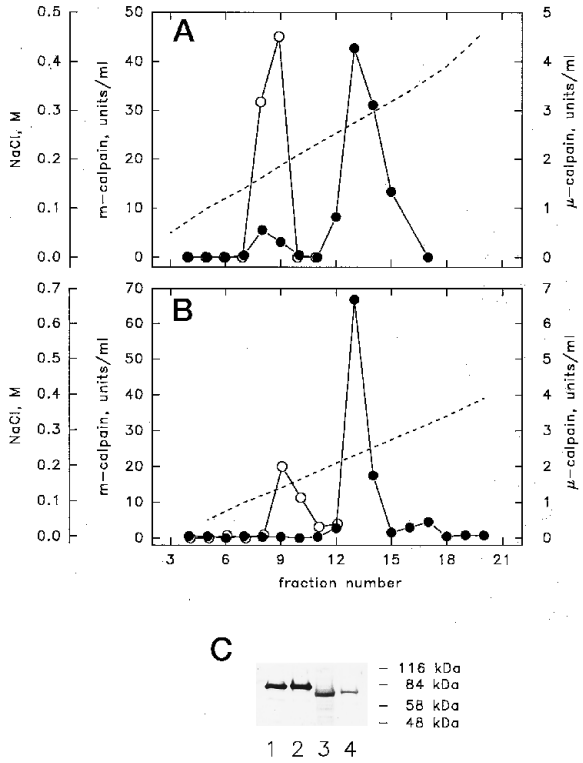

FIG. 5. **DEAE-Sepharose chromatography of calpain fractions obtained by gel filtration of CHO^P or SHI cell homogenate supernatants.** Calpain-containing Bio-Gel fractions from chromatography runs of CHO^P cell (*panel A*) or SHI cell (*panel B*) homogenate supernatants were pooled and analyzed by DEAE-Sepharose chromatography as described under "Experimental Procedures." Fractions were assayed for μ-calpain (*open circles*) or m-calpain (*filled circles*). The *dashed lines* indicate NaCl concentration gradients. *Panel C*, protein immunoblot analysis was carried out on pooled μ- and m-calpain peaks from the DEAE chromatography fractions as described under "Experimental Procedures." *Lanes 1* and *2*, m-calpain fractions; *lanes 3* and *4*, μ-calpain fractions. *Lanes 1* and *3*, CHO^P samples; *lanes 2* and *4*, SHI samples.

pooled μ-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 μ-calpain, *panel A*, fractions 8 and 9 were pooled. For SHI μ-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 μ-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 μ-calpain peak failed to show any substantial alteration in calcium requirement ($A_{0.5}$ for Ca^{2+} = 28 μM for SHI calpain and 41 μM for CHO^P calpain) and sensitivity to inhibition by ZLLY-CHN$_2$ ($I_{0.5}$ = 0.11 μM for SHI calpain and 0.18 μM for CHO^P calpain) or by calpastatin ($I_{0.5}$ = 2.9 pmol for SHI calpain and 3.8 pmol of calpastatin/ml

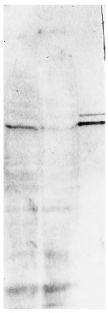

FIG. 6. **Accumulation of human μ-calpain in electroporated SHI cells.** SHI cells were electroporated in the presence of 2 mg of human μ-calpain/ml and then analyzed for μ-calpain large subunit by Western blotting as described under "Experimental Procedures." A control sample of cells was treated under 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 μ-calpain.

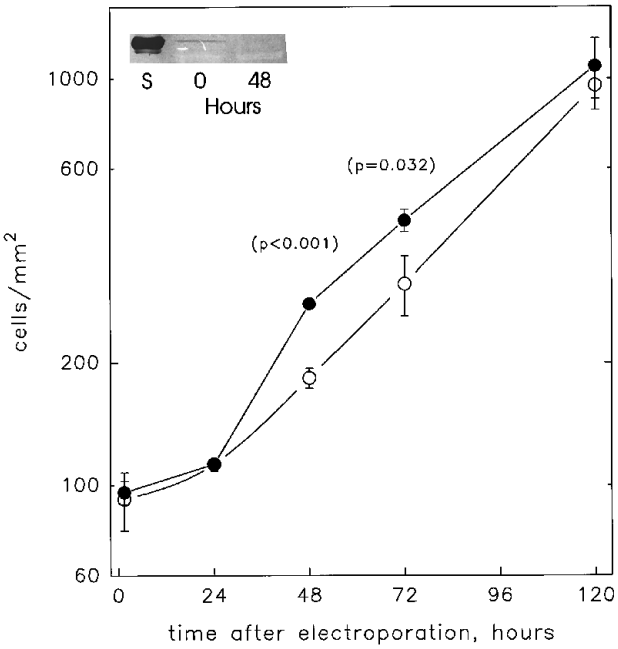

FIG. 7. **Introduction of human μ-calpain into SHI cells increased growth rate.** SHI cells were electroporated in the presence of 2 mg of μ-calpain/ml (*filled circles*) or 2 mg of BSA/ml (*open circles*) and then plated in wells as described under "Experimental Procedures." At various times after electroporation, cell counts were taken. *Error bars* indicate mean ± 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*, μ-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 3×10^5 cells were electrophoresed and analyzed by immunoblotting for human μ-calpain. *S*, 800 ng of human μ-calpain large subunit.

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

Restoration of Wild-type Growth Rate by Electroporation of μ-Calpain into SHI Cells—When SHI cells were subjected to electroporation in the presence of 2 mg of human μ-calpain/ml, as described under "Experimental Procedures," they accumulated approximately 25 ng of μ-calpain/10^6 cells (Fig. 6). This is nearly the same as the content of μ-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 μ-calpain (5.4 units/μg protein), and the μ-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 30×10^6 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 μ-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 μ-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 μ-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 ± 8 and 199 ± 64 (S.D.) cells/mm^2 were obtained for cells electroporated in the presence of μ-calpain and BSA, respectively (n = 3 culture wells, p = 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 μ-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.

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 μ-calpain was approximately as sensitive as CHO^p μ-calpain to inhibition by ZLLY-CHN_2. Moreover, the μ-calpain present in SHI cells did not appear to differ significantly from CHO^p μ-calpain in chromatographic properties (Figs. 4 and 5), large subunit molecular mass (Fig. 5, *panel C*), Ca^{2+}-requirement, or sensitivity to inhibition by calpastatin. These results indicate that SHI cells express a form of μ-calpain that is similar to, or identical with, wild-type μ-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 μ-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 μ-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-

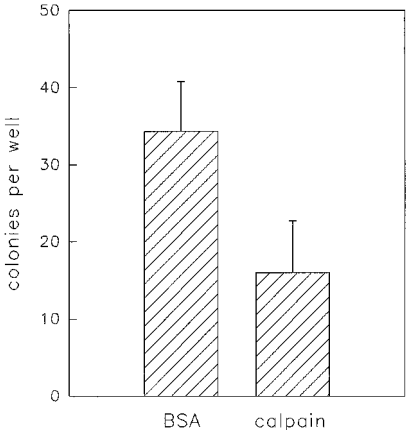

FIG. 8. **SHI cells were electroporated in the presence of BSA or human μ-calpain, and 1000 viable cells were plated in 9.5 cm² culture plate wells for cell survival assay as described under "Experimental Procedures."** After 24 h ZLLY-CHN_2 was added for 16 additional h, and the cells were then cultured in the absence of ZLLY-CHN_2 until visible colonies formed. *Error bars*, standard deviations ($n = 6$ wells). The calpain sample is statistically different from the BSA sample at the $p < 0.001$ level.[/fig]

ration of purified μ-calpain into SHI cells decreased their survival rate upon exposure to ZLLY-CHN_2 (Fig. 8). It should be noted that more μ-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 μ-calpain on growth rate ends. This may allow some cells to escape from the toxic effects of ZLLY-CHN_2 before the 16-h treatment is over. There is also the likelihood that electroporation did not result in a uniform distribution of μ-calpain in the cells. Non-uniform uptake following electroporation of antibodies into mammalian cells has been noted (38). Therefore, some cells may not have accumulated sufficient amounts to greatly alter their susceptibility to ZLLY-CHN_2 toxicity.

SHI cells proliferated at a lower rate than the wild-type CHO^P cells, as predicted by the hypothesis that calpains are required for regulation of cell proliferation. The increase in population doubling time from 19 to 29 h (Fig. 2) could be entirely accounted for by a prolongation of G_1 phase by approximately 10 h (Fig. 3).

Injection of purified human erythrocyte μ-calpain into SHI cells by electroporation increased the growth rate of these cells over the first few population doublings (Fig. 7). The transitory nature of this effect would be expected with catabolism of the human μ-calpain within SHI cells and its dilution between two daughter cells with each cell division. As indicated in the *inset* for Fig. 7, there was a marked reduction in human μ-calpain content per SHI cell 48 h after electroporation. The increased growth rate was not the result of electroporation alone, since electroporation in the presence of an "inert" protein, BSA, had no effect (Fig. 7, *open circles*). Moreover, exposure of the SHI cells to the same combination of calpain and calpastatin utilized in these studies, but without electroporation, did not alter cell growth (not shown), indicating that the accelerated growth rate was not the result of extracellular effects of calpain or calpastatin. The ability to temporarily restore the defect in growth rate by introduction of exogenous μ-calpain is consistent with the idea that a decreased content of this isozyme is responsible for the slow growth of SHI cells.

Calpains and Cell Division—Several different laboratories have presented observations consistent with the participation of calpains in cell division (24, 42, 43). Ours is the first study to suggest a specific role for the μ-calpain isozyme in cell cycle progression. Previously, it was suggested that microinjection of m-calpain near nuclei of PtK_1 cells accelerated progression through M phase (42), whereas μ-calpain reportedly did not influence mitosis. The present studies indicate that μ-calpain is important for the rate of progression through G_1 phase. Thus, calpains appear to be involved in at least two different phases of the cell cycle, G_1 and M. Consistent with this notion, studies of the effects of cell-permeant calpain inhibitors on cell cycle progression of synchronized smooth muscle cells have indicated blockade in both G_1 and G_2/M (43). Although it is possible that the two different calpain isozymes function independently, m-calpain in M phase and μ-calpain in G_1 phase, we have not ruled out a role for m-calpain in the latter.

In this study, we have applied the hypothesis that calpains are targets for the antiproliferative effect of the cysteine protease inhibitor, ZLLY-CHN_2, to isolate a μ-calpain-deficient CHO^P cell clone which also has an increased population doubling time. The wild-type proliferative growth rate could be temporarily restored by microinjection of purified μ-calpain, providing evidence that this calpain isozyme has a role in regulating proliferation of SHI cells. Our observations are consistent with the hypothesis that μ-calpain is involved in setting the duration of the G_1 to S phase interval and establishes a system for testing this idea. Further studies to establish this hypothesis are ongoing, and a caveat that should not be ignored is the possibility that the intracellular target for ZLLY-CHN_2 is not μ-calpain but a related protein. The studies presented in this article argue that the target is at least very similar in properties to μ-calpain, since ZLLY-CHN_2 is an active site-directed inhibitor of cysteine proteases with very little reactivity toward sulfhydryls in general (23), and the defect in SHI cells could be reversed by microinjection of purified μ-calpain. More information could be gained by molecular genetic studies, but, unfortunately, the cDNAs for hamster calpains are not available. We have recently found that antisense phosphorothioate oligodeoxynucleotides against human calpain small subunit will deplete calpain in cultured human cells, and inhibit cell growth.[2] This observation lends support to the possibility that similar studies with the SHI cells will provide evidence for the role of calpains in their growth. One such study would be stable transfection of SHI cells with μ-calpain cDNA to determine whether this will produce reversion to the wild-type growth phenotype. In summary, by comparing the SHI and parental CHO^P cell lines at the cell physiological and biochemical levels, it may be possible to gain a detailed understanding of how μ-calpain influences proliferative cell growth and perhaps other cellular functions.

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REFERENCES

1. Norbury, C., and Nurse, P. (1992) *Annu. Rev. Biochem.* **61,** 441–470
2. Murray, A., and Hunt, T. (1994) *The Cell Cycle*, pp. 106–116, Freeman, New York
3. Murray, A. W., Solomon, M. J., and Kirschner, M. W. (1989) *Nature* **339,** 280–286
4. Minshull, J., Pines, J., Golsteyn, R., Standart, N., Mackie, S., Colman, A., Blow, J., Ruderman, J. V., Wu, M., and Hunt, T. (1989) *J. Cell Sci.* **12,** (suppl.) 77–97
5. Heck, M. M. S., Hittleman, W. N., and Earnshaw, W. C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1086–1090
6. Brown, K. D., Coulson, R. M. R., Yen, T. J., and Cleveland, D. W. (1994) *J. Cell Biol.* **125,** 1303–1312
7. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990) *Cell* **63,** 1129–1136
8. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) *Nature* **349,** 132–138

[2] Zhang, W., Lane, R. D., and Mellgren, R. L., *J. Biol. Chem.*, in press.

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