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Proteolysis of Nuclear Proteins by μ-Calpain and m-Calpain*

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Purified calpains are capable of proteolyzing several high M_r nuclear proteins and solubilizing a histone H1 kinase activity from rat liver nuclei upon exposure to 10^{-6} – 10^{-5} M Ca^{2+}. Major nuclear substrates displayed apparent molecular masses of 200, 130, 120, and 60 kDa on Coomassie Blue-stained SDS-PAGE gels. The nuclear proteins and the H1 kinase were released from Triton-treated nuclei following incubation with buffer containing 0.5 M NaCl. They therefore appeared to be internal nuclear matrix proteins. The nuclear H1 kinase activity solubilized by incubation with m-calpain was eluted in the void volume of a Bio-Gel A-1.5m column, indicating an apparent mass greater than 1,500 kDa. Treatment of the calpain-solubilized kinase with 0.5 M NaCl dissociated it to a form having an apparent mass of 300 kDa (Stokes radius = 5.6 nm), suggesting that the 300-kDa kinase is released from nuclei by calpain treatment as a large complex containing other internal matrix proteins. Purified human erythrocyte μ-calpain was capable of proteolyzing the nuclear matrix proteins at 10^{-6} M Ca^{2+}. In contrast, human erythrocyte multicatalytic protease complex produced little cleavage of the nuclear proteins. Proteolysis of nuclear proteins by either μ-calpain or m-calpain was inhibited by calpastatin. These experiments suggest a physiologic role for the calpains in the turnover of nuclear proteins.

Although it is recognized that all proteins in cells have finite lifespans, and that intracellular proteins are ultimately removed from cells by the process of proteolysis, surprisingly little is known about the specific proteases involved in the turnover of individual proteins.

Several major cytoplasmic proteases have now been described, purified, and extensively studied. The multicatalytic protease complex, MPC,[1] (1-3) appears to function in the turnover of denatured intracellular proteins (2, 4). It is widely distributed in various tissues and has been reported to comprise about 1% of the soluble protein in rat liver (5). The calpains are ubiquitously distributed thiol proteases which have an absolute requirement for Ca^{2+} (6-8). The intracellular functions of the calpains have not been defined, although there is some evidence that they may be involved in the turnover of cytoskeletal (9, 10) or cellular membrane-associated (11) proteins. A major problem in defining the role of the m-calpain isozyme has been its apparent requirement for 1 mM Ca^{2+} (6, 12), which is several orders of magnitude higher than intracellular free Ca^{2+} concentrations.

One physiologic function of the intracellular proteases may be regulation of the cell cycle. Various protease inhibitors are known to arrest cell division (13, 14), and the rapid proteolysis of cyclin at the metaphase/anaphase transition is a well documented, critically important event in mitosis (15, 16). Recently, a number of observations have suggested that calpains may play a role in cell division. It has been reported that m-calpain immunoreactivity became associated with chromosomes during mitosis in PtK_1 cells and that mitosis was accelerated by microinjection of m-calpain, and arrested by microinjection of calpastatin, the highly specific calpain inhibitor protein (17). Rapid proteolysis of the c-*mos* gene product, $pp39^{mos}$, occurred after *xenopus* oocyte fertilization, and appeared to be catalyzed by m-calpain (18).

Other experiments employing isolated nuclei suggest that calpains may have a function in nuclear protein turnover. An endogenous, Ca^{2+}-activated, leupeptin-sensitive protease activity proteolyzed high molecular weight matrix proteins in rat liver nuclei prepared in the absence of protease inhibitors (19). Recent preliminary studies have shown that m-calpain in the presence of 10^{-6}-10^{-5} M Ca^{2+} could solubilize a histone H1 kinase activity from rat liver nuclei (20).

In the present study, the major nuclear protein substrates of μ-calpain and m-calpain are determined, and shown to be sensitive to cleavage by either calpain isozyme at physiologic Ca^{2+} concentrations.

EXPERIMENTAL PROCEDURES

Materials—Triton X-100, EGTA, HEDTA, bovine pancreatic DNase I, bovine pancreatic RNase A (Type I-AS), porcine thyroglobulin, bovine liver catalase, whale muscle myoglobin, prestained molecular weight markers for SDS-PAGE (MW-SDS-Blue), and rabbit liver glycogen were obtained from Sigma. Phenyl-Sepharose, a product of Pharmacia LKB Biotechnology Inc., was purchased from Sigma. Acrylamide, bisacrylamide, and all other chemicals utilized in electrophoresis, protein blotting, and immunostaining were obtained from Bio-Rad. Bio-Gel A-1.5m was also purchased from Bio-Rad. Reagent-grade calcium acetate was obtained from Baker. Leupeptin was purchased from Chemicon. Calf thymus histone H1, purified by a standard method (21), was kindly provided by Dr. Keith Schlender, from the Department of Pharmacology at this institution.

Calcium Buffers—Determination of Ca^{2+} requirements of μ-calpain and m-calpain on nuclear protein substrates were carried out in the presence of Ca^{2+}·EGTA, or, where indicated, Ca^{2+}·HEDTA buffers. Chelex-treated water was used in all buffers. In either buffer system, the total chelator concentration was 2.00 mM, and aliquots of a stock calcium acetate solution were added to produce the desired $[Ca^{2+}]_f$ based on the apparent stability constants of the Ca^{2+}-ligand complex at pH 7.0 (22). The buffers were retitrated to pH 7.0 after addition of calcium. Free calcium concentrations of the final buffered solutions were checked with a Ca^{2+}-selective Kwik-Tip electrode, and Ultrawick reference electrode (World Precision Instruments), using CAL BUF calcium standards (World Precision Instruments). All reported Ca^{2+} readings are based on results obtained with the calcium electrode, which agreed within 20% with the $[Ca^{2+}]_f$ calculated from the stability

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[1] The abbreviations used are: MPC, multicatalytic protease complex; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEDTA, *N*-hydroxyethylethylenediaminetetraacetic acid; $[Ca^{2+}]_f$, free Ca^{2+} concentration; Mops, 4-morpholinepropanesulfonic acid.

constants over the appropriate buffering ranges for EGTA (10^{-7}-10^{-6} M Ca^{2+}) and HEDTA (10^{-6}-10^{-5} M Ca^{2+}).

Preparation of Proteases and Calpastatin—All final preparations were at least 90% pure by densitometric analysis of Coomassie Blue-stained SDS-PAGE gels.

Bovine myocardial *m*-calpain was prepared as previously described (23), except that the final reactive-red agarose chromatography step was replaced by Bio-Gel A-1.5m and phenyl-Sepharose chromatography steps as follows. All procedures were carried out in the cold (4–7 °C). The ammonium sulfate precipitate from the penultimate purification step was dialyzed against column buffer (50 mM imidazole-HCl, 1 mM EDTA, 1 mM EGTA, 80 mM NaCl, 1 mM dithiothreitol, pH 7.8), and applied in a volume of 20 ml to a 2.5- × 120-cm column of Bio-Gel A-1.5m equilibrated in column buffer. The pooled calpain fractions were then adjusted to 0.3 M NaCl and applied to a 50-ml column of phenyl-Sepharose equilibrated in column buffer containing 0.3 M NaCl. The column was washed with 150 ml of column buffer containing 0.3 M NaCl, and *m*-calpain was eluted with a 200-ml linear gradient of column buffer plus 0.3 M NaCl (start buffer) and 5 mM EGTA, 1 mM dithiothreitol, pH 7.8 (end buffer).

Human erythrocyte μ-calpain was purified from 500–600 ml of outdated blood by a procedure similar to the one described above for *m*-calpain. The erythrocytes were washed three times with isotonic saline and then lysed with 4 mM EGTA, 1 mM dithiothreitol, pH 7.4. After centrifugation at 12,000 × *g* for 10 min, the lysate was applied to a 50-ml column of DEAE-Sepharose equilibrated with column buffer containing 0.09 M NaCl. The column was washed with 150 ml of column buffer containing 0.09 M NaCl, and μ-calpain was eluted with column buffer containing 0.18 M NaCl. Fractions containing μ-calpain were pooled and subjected to Bio-Gel and phenyl-Sepharose chromatography as described for *m*-calpain purification, except that the sample and phenyl-Sepharose column equilibration buffer contained 0.15 M NaCl instead of 0.3 M NaCl.

Human erythrocyte MPC (24) and bovine myocardial calpastatin (25) were purified by previously described methods.

Preparation of Rat Liver Nuclei—Rat liver nuclei were prepared by a modification of an established method (26). 1 mM dithiothreitol, 10 μM leupeptin, 1 mM benzamidine, 2 μM pepstatin A, and 0.25 mM phenylmethanesulfonyl fluoride were included in all buffers. The nuclei were stored at −70 °C in storage buffer: 50 mM Tris-HCl, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 2 μM pepstatin A, 1 mM benzamidine, 10 μM leupeptin, pH 7.5 measured at 23 °C. Nuclei were stored in 0.5 ml buffer per g of liver tissue processed.

Prior to incubation with proteases, purified nuclei were further washed at ambient temperature two times with 50 mM Tris-HCl, 5 mM magnesium acetate, 20 μM leupeptin, 1 mM dithiothreitol, 0.5% Triton X-100, pH 7.5, and at least three times with 50 mM Mops, 1 mM magnesium acetate, 1 mM dithiothreitol, pH 7.0, containing Ca^{2+}·EGTA buffers. Calcium concentrations were measured in the buffers prior to incubation with nuclei. In separate experiments it was determined that washing the nuclear samples several times with Ca^{2+} buffers as described above was sufficient to remove any endogenous $[Ca^{2+}]_f$ contributed by the nuclei. That is, the $[Ca^{2+}]_f$ concentration in the last postnuclear supernatant was virtually identical to the $[Ca^{2+}]_f$ in the stock buffer, as measured with the Ca^{2+} electrode. The nuclear preparations were finally resuspended in a volume of Ca^{2+} buffer equivalent to the initial volume of stored nuclear preparation taken for the experiment.

In experiments employing Ca^{2+}·HEDTA buffers, Mg^{2+} was eliminated from the wash buffers since it competes with Ca^{2+} for binding to HEDTA.

Treatment of Nuclei with Proteases—Washed nuclei were incubated with calpains or MPC at 25 °C for the indicated period of time. Constant agitation was necessary to insure uniform suspension of the nuclei, and this was provided by carrying out the incubation in a shaker bath operating at 280 excursions per min. In experiments designed to assess proteolysis of nuclear proteins, the incubation was terminated by addition of SDS-PAGE sample buffer immediately followed by heating to 100 °C for 5 min. Samples were then subjected to SDS-PAGE in 8% polyacrylamide gels using the Laemmli buffer system (27). Proteolysis of nuclear proteins was quantitated by scanning Coomassie Blue-stained protein bands having molecular masses >100 kDa using a Zeineh laser densitometer and integrating the peak areas. Loss of protein bands was determined by comparing the peak areas of treated samples with peak areas of untreated controls applied to the same gel slab.

Release of Nuclear Histone H1 Kinase by Calpains—Nuclei were pelleted by centrifugation at 12,000 × *g* after incubation with calpains as described above, but without addition of SDS-PAGE sample buffer. The supernatants were then adjusted to 5 mM EGTA and aliquots were assayed for histone H1 kinase activity in the presence of 5 mM EGTA as previously described (20, 28). Briefly, 5-μl aliquots of kinase were incubated at 30 °C with 0.25 mg of histone H1 per ml in 50 mM Tris-HCl, 5 mM EGTA, 5 mM magnesium acetate, 0.1 mM [γ-^{32}P]-ATP (100–1000 cpm/pmol), pH 7.5. After a 5-min incubation, 25-μl aliquots of reaction mixture were pipetted onto 1-cm^2 patches of phosphocellulose paper and washed extensively with 50 mM NaCl. The papers were then washed with acetone, dried, and counted in a liquid scintillation counter. A unit of histone kinase activity incorporates 1 pmol of phosphate into histone H1 per min.

Gel Filtration of Calpain-released Kinase—A 1-ml sample of washed nuclei was incubated with 30 μg of *m*-calpain per ml and 100 μM $[Ca^{2+}]_f$ for 30 min at 25 °C. All further procedures were carried out at 4–7 °C. Nuclei were then removed by centrifugation for 10 min at 12,000 × *g*, and 5 mM EGTA was added to the supernatant, along with 2 mg each of catalase and myoglobin as marker proteins. The supernatant was applied to a 1- × 32-cm column of Bio-Gel A-1.5m equilibrated in 50 mM Tris-HCl, 5 mM EGTA, 1 mM dithiothreitol, 100 mM NaCl, pH 7.5, measured at 23 °C. The column flow rate was 4 ml per h, and 0.5-ml fractions were collected and assayed for histone kinase activity as described above. A control sample was chromatographed separately under the same conditions. It consisted of the supernatant of the nuclear wash step directly prior to calpain treatment of the nuclei.

Preparation of Nuclear Subfractions—Isolated nuclear preparations (0.5 ml) were thawed and washed once with storage buffer. DNase I (0.25 mg/ml) and RNase A (0.25 mg/ml) were added to the tubes at 4 °C and the samples were incubated for 60 min. The nuclease-treated nuclei were then centrifuged at 10,000 × *g* for 10 min, and the supernatants saved (S1 fraction). The pellets were homogenized in 0.5 ml of nuclear wash buffer (50 mM Tris-HCl, 1 mM magnesium acetate, 0.1 M NaCl, 1 mM dithiothreitol, pH 7.4, measured at 23 °C) and centrifuged again. The supernatant will be referred to as the 0.1 M NaCl fraction. The pellet from this step was sequentially washed with nuclear wash buffer containing 0.5 M NaCl and 1.6 M NaCl. The supernatants from these steps will be called the 0.5 M NaCl fraction and 1.6 M NaCl fraction, respectively. The pellet after the 1.6 M NaCl wash was homogenized in 0.5 ml of nuclear wash buffer, and is referred to as the 1.6 M NaCl pellet fraction.

RESULTS

m-Calpain-dependent Proteolysis of Rat Liver Nuclear Proteins—Incubation of Triton X-100-washed rat liver nuclei with *m*-calpain in the presence of 30 μM $[Ca^{2+}]_f$, as described under "Experimental Procedures," resulted in proteolytic cleavage of several nuclear proteins at a mass of >50 kDa (Fig. 1, *lane 2*). Lower M_r proteins were not significantly

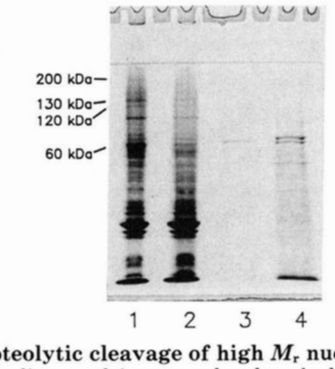

FIG. 1. **Proteolytic cleavage of high M_r nuclear proteins by *m*-calpain.** Rat liver nuclei, prepared and washed as described under "Experimental Procedures" were incubated in the presence of 30 μM $[Ca^{2+}]_f$, and in the presence (*lanes 2* and *4*) or the absence (*lanes 1* and *3*) of 15 μg *m*-calpain/ml. After 30 min at 25 °C, the samples were centrifuged at 12,000 × *g* for 5 min. The supernatants (*lanes 3* and *4*) and pellets (*lanes 1* and *2*) were suspended in SDS-PAGE sample buffer, electrophoresed in 8% polyacrylamide gels, and stained with Coomassie Blue. The molecular masses of calpain substrates were determined by comparison with protein standards run on the same gel.

cleaved. Concomitant with the proteolysis of the sedimentable nuclear proteins, low M_r fragments were solubilized (Fig. 1, *lane 4*). Major substrates for *m*-calpain included a doublet at 200 kDa and other bands at 130, 120, and 60 kDa. Another band at 100 kDa was also proteolyzed in some experiments (Fig. 1). However, in a number of experiments it was clearly less sensitive to cleavage by calpain than the other nuclear proteins (not shown).

Calcium Requirement for Proteolysis of the Nuclear Proteins—Nuclei were incubated with *m*-calpain in buffers containing increasing $[Ca^{2+}]_f$, as described under "Experimental Procedures." Half-maximal proteolysis of the nuclear proteins was noted at a Ca^{2+} concentration of approximately 10^{-5} M (Fig. 2, *closed circles*). In Coomassie Blue-stained gels, there appeared to be little proteolysis of high M_r nuclear proteins at 3 μM $[Ca^{2+}]_f$ (Fig. 2). However, analysis of nuclear protein breakdown by SDS-PAGE followed by the more sensitive silver staining technique (29) revealed a significant increase in proteolytic fragments at this Ca^{2+} concentration (not shown).

All of the experiments conducted to this point had been with nuclei which were extensively washed with Triton X-100 (see "Experimental Procedures"), and therefore depleted of lipids. It was of interest to determine whether phospholipids associated with the nucleus might further decrease the Ca^{2+} requirement for *m*-calpain, since interaction with acidic phospholipids can significantly decrease its Ca^{2+} requirement for proteolysis of other substrates (8). As indicated in Fig. 2, omission of the Triton washes in the nuclear preparation had no predominant effect on the Ca^{2+} requirement for *m*-calpain.

Proteolytic Solubilization of a Nuclear Histone H1 Kinase Activity—Fractionation of nuclear proteins by nuclease treatment and salt extraction as described under "Experimental Procedures" reveals that the major fraction of Ca^{2+}-independent histone H1 kinase activity (assayed as described under "Experimental Procedures") was released from nuclei by extraction with NaCl concentrations of 0.5 M or greater (Table I). A minor fraction of kinase activity was released by nuclease treatment (S1 fraction, Table I). However, nuclease treatment of the extensively washed nuclear preparations utilized in the calpain studies did not result in measurable release of kinase activity (not shown). The high M_r calpain substrates were also solubilized at high salt concentrations (Fig. 3). These properties indicate that the calpain substrate proteins and the calpain-solubilized H1 kinase were associated with the "internal nuclear matrix" (30), which is extractable with high salt concentrations provided that due care has been exercised to prevent oxidation of nuclear thiol groups. These results further suggest the possibility that the kinase was solubilized upon proteolysis of internal matrix proteins by *m*-calpain.

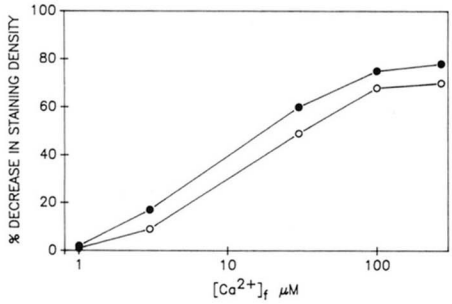

FIG. 2. **Calcium requirement for proteolysis of nuclear proteins by *m*-calpain.** Nuclei were washed as described under "Experimental Procedures" with buffer containing various Ca^{2+} concentrations. *m*-Calpain was added to a final concentration of 15 μg/ml, and the mixture was incubated at 25 °C for 20 min. 10-μl aliquots were subjected to SDS-PAGE and stained with Coomassie Blue. The samples were analyzed for a decrease in staining intensity of high M_r proteins as described under "Experimental Procedures" (●). In another experiment, a nuclear preparation which was not subjected to Triton washing was incubated under the same conditions (○).

TABLE I

Fractionation of histone H1 kinase activity in Triton-washed rat liver nuclei

Samples of nuclei were sequentially incubated with nuclease and NaCl-containing buffers, and the derived supernatants were assayed for histone H1 kinase activity. Details for the procedures are provided in the Methods section.

Fraction	H1 kinase units recovered[a]	% total kinase
S1	48.4 ± 1.6	17.5
0.1 M NaCl	45.1 ± 12.4	16.3
0.5 M NaCl	152 ± 33	54.8
1.6 M NaCl	28.1 ± 10.5	10.1
1.6 M NaCl pellet	3.6 ± 2.4	1.3

[a] Mean ± standard deviation of results obtained with nuclear preparations from four different groups of rats.

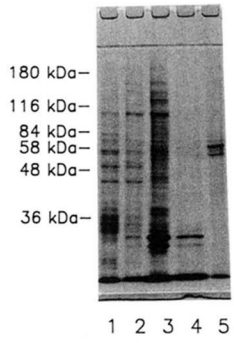

FIG. 3. **Fractionation of nuclear proteins by nuclease and NaCl extraction.** Nuclei were incubated sequentially with nucleases and buffers containing different NaCl concentrations as described under "Experimental Procedures." 10-μl aliquots of the supernatants were electrophoresed in 8% SDS-PAGE slab gels, and stained with Coomassie Blue dye. *Lane 1*, S1 fraction; *lane 2*, 0.1 M NaCl fraction; *lane 3*, 0.5 M NaCl fraction; *lane 4*, 1.6 M NaCl fraction; *lane 5*, 1.6 M NaCl pellet.

A 12,000 × *g* supernatant from *m*-calpain-treated nuclei was applied to a Bio-Gel A-1.5m column, and the eluate was assayed for histone H1 kinase activity (Fig. 4*A*, *closed circles*). Two peaks of kinase activity were eluted; one in the void volume, and another between the marker proteins thyroglobulin (Stokes radius = 8.2 nM) and catalase (Stokes radius = 5.2 nm). The lower M_r peak of kinase was evident, although somewhat reduced in content, in a control sample of extract from nuclei not subjected to calpain treatment (Fig. 4*A*, *open circles*). On the other hand, the excluded kinase peak was barely evident in the control sample.

Treatment of the excluded calpain-released kinase peak with 0.5 M NaCl decreased its apparent molecular mass to 300 kDa (Stokes radius = 5.6 nm), as revealed by rechromatography on Bio-Gel (Fig. 4*B*, *open triangles*). When washed nuclei were extracted with 0.5 M NaCl without prior treatment with calpain, a similar peak of histone H1 kinase activity was solubilized (Fig. 4*B*, *closed triangles*).

Cleavage of Nuclear Proteins by MPC and μ-Calpain—It was of interest to determine whether the other two major intracellular, nonlysosomal proteases had activity on the nuclear proteins. Incubation of nuclei with purified human erythrocyte MPC for up to 1 h under the standard assay conditions did not produce significant proteolysis of nuclear proteins (Fig. 5, *lanes 1–4*). Addition of the potent activator

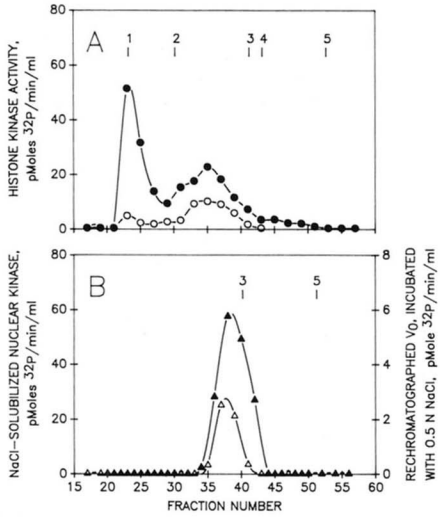

FIG. 4. **Gel filtration of calpain-released nuclear kinase.** Histone H1 kinase activity was released from nuclei by *m*-calpain and subjected to gel filtration analysis on a Bio-Gel A-1.5m column as described under "Experimental Procedures" (*A*, ●). An untreated control sample (see "Experimental Procedures") was chromatographed separately (○). In other experiments, the excluded calpain-released kinase (*tube 22* in *A*) was adjusted to contain 0.5 M NaCl and rechromatographed in the presence of 0.5 M NaCl (*B*, △). A 1.0-ml sample of washed nuclei was extracted with buffer containing 0.5 M NaCl, centrifuged at 12,000 × *g* for 10 min, and the supernatant was applied to the column (*B*, ▲). The markers and their Stokes radii were: *1*, liver glycogen (V_0); *2*, thyroglobulin, 8.2 nm; *3*, catalase, 5.2 nm; *4*, *m*-calpain, 4.4 nm; *5*, myoglobin, 1.9 nm.

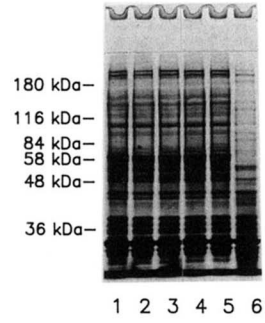

FIG. 5. **Incubation of nuclei with MPC and μ-calpain.** Nuclei were washed in buffer containing 3 μM $[Ca^{2+}]_f$, and incubated as described under "Experimental Procedures" with 30 μg of MPC/ml or 15 μg of μ-calpain/ml. *Lane 1*, untreated nuclei; *lane 2*, incubated with MPC for 20 min; *lane 3*, incubated with MPC for 60 min; *lane 4*, incubated with MPC and 50 μM protamine for 60 min; *lane 5*, incubated with μ-calpain in the presence of 5 mM EGTA for 20 min; *lane 6*, incubated with μ-calpain for 20 min. The M_r values on the *left* indicate the mobilities of the prestained molecular weight markers included on the same gel.

protamine (24) did not result in increased proteolysis by MPC (Fig. 5, *lane 4*). In contrast, incubation of nuclei with purified human erythrocyte μ-calpain extensively proteolyzed the same proteins which were excellent *m*-calpain substrates (Fig. 5, *lane 6*). Half-maximal cleavage of the high M_r proteins and half-maximal release of H1 kinase occurred at approximately 2 μM $[Ca^{2+}]_f$ (Fig. 6).

Calpastatin Inhibition of Calpain-catalyzed Proteolysis of Nuclear Proteins—Bovine myocardial calpastatin is a tightly binding inhibitor which is highly selective for the calpains (6, 8). Inhibition by calpastatin is currently the best evidence that a proteolytic cleavage is catalyzed by a calpain.

Proteolysis of nuclear proteins by μ-calpain (Fig. 7*A*) or *m*-calpain (Fig. 7*B*) was inhibited in a concentration-dependent

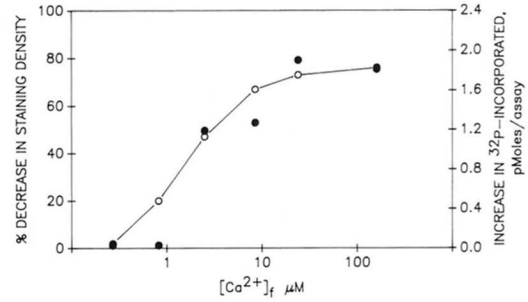

FIG. 6. **Ca^{2+} requirement for the proteolysis of nuclear proteins and the release of histone kinase by μ-calpain.** Washed nuclei were incubated with 15 μg of μ-calpain/ml in the presence of various concentrations of $[Ca^{2+}]_f$. In this experiment, HEDTA was used as a Ca^{2+} buffer between 1 and 30 μM $[Ca^{2+}]_f$, and no Mg^{2+} was included in the incubation buffers. Proteolysis of nuclear proteins (○) and release of histone kinase activity (●) were determined as described under "Experimental Procedures."

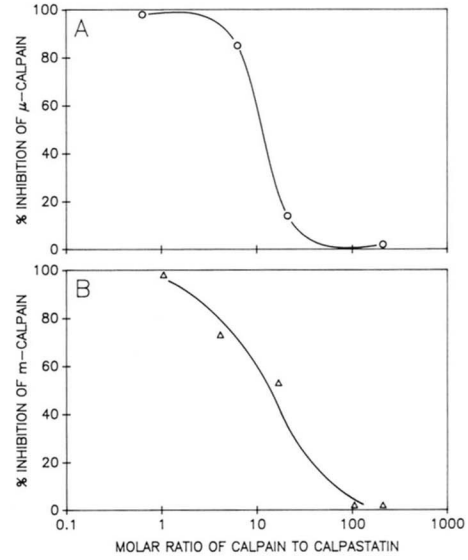

FIG. 7. **Calpastatin inhibition of μ-calpain- and *m*-calpain-catalyzed proteolysis of nuclear proteins.** Nuclei were incubated with 15 μg of μ-calpain/ml (*A*) or 30 μg of *m*-calpain/ml (*B*) in the presence of various concentrations of bovine myocardial calpastatin. After 20 min, aliquots of the samples were subjected to SDS-PAGE, and the proteolysis of nuclear proteins was quantitated as described under "Experimental Procedures."

manner by purified bovine myocardial calpastatin. Bovine myocardial calpastatin and other large calpastatins, contain four independently functioning inhibitory sites for calpains (8). Therefore, at a molar ratio of eight calpains per calpastatin molecule, there should be a 50% reduction in calpain activity. As indicated in Fig. 7, half-maximal inhibition of μ-calpain and *m*-calpain occurred near this theoretical value. Thus, calpastatin appeared to be an efficient inhibitor of nuclear protein cleavage by either calpain isozyme.

DISCUSSION

Preliminary studies from this laboratory indicate that *m*-calpain could solubilize a nuclear protein kinase at $[Ca^{2+}]_f$ between 10^{-6} and 10^{-5} M (20). The present investigation was undertaken in part to determine whether this was a specific effect of *m*-calpain on the kinase, and to determine whether the other major defined cytoplasmic proteases may also proteolyze nuclear proteins.

The nuclear action of *m*-calpain did not appear to be limited to the histone H1 kinase, since it could cleave several nuclear

proteins at 10^{-5} M $[Ca^{2+}]_f$ (Figs. 1 and 2). Furthermore, solubilization of histone H1 kinase activity by *m*-calpain did not appear to be the result of direct cleavage of the kinase. The major Ca^{2+}-independent histone H1 kinase activity in the extensively washed nuclei employed in these studies appeared to be associated with the salt-extractable, internal nuclear matrix (Table I, and "Results"). The experiments described in Fig. 4 indicate that calpain-solubilized H1 kinase activity is associated with extremely large complexes of matrix proteins released from the nuclei by calpain treatment. Further incubation of this large complex with 0.5 M NaCl resulted in dissociation of the kinase to the 5.6-nm form evident in 0.5 M NaCl extracts of nuclei (Fig. 4*B*). Thus, treatment with calpain did not appear to produce a lower molecular weight form of the kinase. The simplest interpretation of these results is that proteolysis results in cleavage and solubilization of matrix proteins, but not their dissociation to noninteracting fragments. Further addition of NaCl to increase the ionic strength results in dissociation of the fragments and release of the 5.6-nm kinase.

μ-Calpain effectively proteolyzed the same nuclear proteins which were sensitive to *m*-calpain, while MPC did not produce significant breakdown (Fig. 5). These results indicate that the nuclear proteins are not intrinsically sensitive to proteolytic cleavage by MPC. However, one cannot rule out the possibility that ubiquitination of the nuclear proteins or interaction of the MPC with various protein factors may allow proteolysis to occur (31).

The results of the present study are consistent with the previously reported proteolysis of high M_r matrix proteins in isolated nuclei by an endogenous Ca^{2+}-stimulated protease activity (19), and strongly suggest that this activity was a calpain. However, calpain immunostaining was not detected in several nuclear preparations (not shown) using a monoclonal antibody named P-1 which recognizes μ-calpain and *m*-calpain small subunit (32). Direct measurement of release of H1 kinase indicates a lack of leupeptin-sensitive, Ca^{2+}-dependent kinase solubilizing activity in extensively washed nuclei (20). Immunofluorescent localization studies in PtK_1 cells indicate that *m*-calpain was not localized in the nucleus of these cells during interphase (17). It may be that calpains are present in the nucleus in small amounts which escape detection by the immunologic techniques employed thus far, or they may be loosely associated, and subsequently released by the washing steps employed in nucleus isolation. It is also possible that they are normally cytoplasmic, and only gain access to the nuclear contents following disruption of the nuclear envelope at the beginning of cell division.

Although calpains may be activated in some experimental protocols by association with acidic phospholipids (8), the mechanism responsible for decreasing the Ca^{2+} requirements of μ-calpain and *m*-calpain when acting on nuclear proteins does not appear to involve binding to phospholipids (Fig. 2). The nuclear calpain substrates may be intrinsically sensitive to calpain activity at low Ca^{2+} concentrations, or there may be a currently unidentified nuclear factor involved in decreasing calpain Ca^{2+} requirement. Resolution of these possibilities will require studies on isolated nuclear proteins as calpain substrates.

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