

m-Calpain Requires DNA for Activity on Nuclear Proteins at Low Calcium Concentrations*

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m-Calpain (calpain II, *m*-CANP), which normally requires millimolar Ca^{2+} for activity *in vitro*, was capable of proteolyzing a number of matrix proteins in isolated rat liver nuclei at Ca^{2+} concentrations as low as 3 μM (Mellgren, R. L. (1991) *J. Biol. Chem.* 266, 13920-13924). Treatment of nuclei with deoxyribonuclease I eliminated the activity of *m*-calpain at low Ca^{2+} concentrations, while ribonuclease A and phospholipase C had no effect. Addition of DNA to DNase-treated nuclei restored *m*-calpain activity at low Ca^{2+} . RNA had little if any effect. Eukaryotic and prokaryotic DNA were equally effective, and synthetic polydeoxyribonucleotides were also activators. *m*-Calpain did not bind to a DNA-cellulose column in the presence of 200 μM Ca^{2+} , and *m*-calpain preincubated in the presence of DNA and 200 μM Ca^{2+} was not activated at low Ca^{2+} concentrations following removal of the DNA. DNA did not alter the Ca^{2+} requirement for *m*-calpain-catalyzed cleavage of casein. These results demonstrate that the Ca^{2+} requirement for proteolysis of nuclear matrix proteins by *m*-calpain can be dramatically decreased in the presence of DNA. Activation did not seem to be a result of DNA binding directly to calpain but appeared to require interaction of DNA, calpain, and calpain substrates in the nuclear matrix.

There is a growing body of evidence for the role of proteolysis in maintaining the orderly transit of cells through the cell cycle (1-4). A number of cell cycle-regulated proteins including cyclin (2), the *c-mos* gene product (4), and DNA topoisomerase II (5) are rapidly degraded during or immediately following cell division. While cyclin proteolysis appears to be catalyzed by the ATP-ubiquitin proteolytic system (6), the protease(s) responsible for degradation of the other proteins have not been identified.

Several observations suggest that the intracellular Ca^{2+} -dependent thiol protease, *m*-calpain (also called calpain II or *m*-CANP) may be involved. Mitosis in cultured PtK₁ cells was reported to be accelerated by microinjection of *m*-calpain (3) or arrested by microinjection of calpastatin (3). The presence of cell-permeable inhibitors of calpains in smooth muscle cell culture medium prevented progression of the cells through S phase (7).

Despite these indications that *m*-calpain may be involved in cell division, a major obstacle in assessing the physiologic

role of *m*-calpain has been its requirement for millimolar Ca^{2+} in proteolyzing various protein substrates *in vitro* (8, 9). However, in a previous communication, proteolysis of several proteins in isolated rat liver nuclei by purified *m*-calpain was shown to require only 10^{-6} to 10^{-5} M Ca^{2+} (10). Proteolysis at low Ca^{2+} was inhibited by addition of leupeptin, or the specific calpain inhibitor calpastatin (10); demonstrating unambiguously that a calpain was required. We now show that *m*-calpain activity on nuclear proteins at these low concentrations of Ca^{2+} is dependent on the presence of endogenous DNA.

EXPERIMENTAL PROCEDURES

Materials—*Bacillus cereus* phospholipase C (Type XIII), pancreatic DNase I (Type II), pancreatic RNase A (Type I-AS), salmon testis DNA (Type III), calf thymus DNA (Type I), *Escherichia coli* DNA (Type VIII), polydeoxyadenylic acid, polythymidylic acid, polydeoxyadenylic acid: polythymidylic acid copolymer, and double stranded DNA-cellulose were obtained from Sigma. Whole yeast RNA was a kind gift from Dr. Susan Dignam in the Department of Biochemistry. It was stored in suspension in ethanol at -20° until needed. Gel electrophoresis supplies were purchased from Bio-Rad.

Buffers—Except where noted, the pH buffer routinely used was buffer A: 50 mM MOPS,¹ 5 mM Mg^{2+} acetate, 1 mM DTT, pH 7.0, at 23°C .

Calcium buffers were utilized whenever free Ca^{2+} concentrations less than 100 μM were required. The calcium buffers were prepared in buffer A containing 2.00 mM EGTA or in buffer B (100 mM MOPS, 1 mM DTT, pH 7.0, at 23°C) containing 2.00 mM *N*-hydroxyethyl-ethylenediamine-*N,N,N'*-triacetic acid. Calcium acetate was added to these solutions to produce the desired free Ca^{2+} concentration. The pH of the buffers was adjusted to 7.0 after the addition of Ca^{2+} . The apparent $\text{p}K_{\text{DS}}$ of the Ca^{2+} -EGTA complex and the Ca^{2+} -*N*-hydroxyethyl-ethylenediamine-*N,N,N'*-triacetic acid complex at pH 7.0 were taken as 6.65 and 5.38, respectively, under the conditions of our studies. The latter values were calculated from the published stability constants (11) and correction factors for ionic strength (12). The accuracy of free Ca^{2+} concentrations based on these apparent stability constants has previously been confirmed in the authors' laboratory by direct measurement with a Ca^{2+} electrode (10). Deionized and reverse-osmosis-purified water was treated by passage through a Chelex 100 column prior to use in calcium buffers.

Nuclear Preparations—Triton X-100-washed nuclei were prepared from rat liver as previously described (10, 13). Nuclei were stored frozen at -70°C in 0.5-ml buffer per g of liver. The storage buffer contained 50 mM Tris-HCl, 0.25 M sucrose, 25 mM KCl, 5 mM MgCl_2 , 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 mM benzamidine, 10 μM leupeptin, pH 7.5, measured at 23°C . Nuclear protein concentrations of the preparations determined by the method of Lowry *et al.* (14) varied from 4.4 to 7.7 mg/ml. DNA content assayed by the diphenylamine assay (15) varied from 3.6 to 4.7 mg/ml.

Protein Preparations—Bovine myocardial *m*-calpain (10) and bovine myocardial calpastatin (16) were purified by established procedures. No μ -calpain was detectable in the *m*-calpain preparation either by direct assay or by immunoblotting with antibodies specific

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¹ The abbreviations used are: MOPS, 4-morpholinopropane sulfonic acid; DTT, dithiothreitol.

for μ -calpain large subunit. Within the limits of detection of the methods used, we estimate that μ -calpain contamination was no more than 0.1% of total calpain.

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide slab gels using the buffer system of Laemmli (17). Gels were stained with Coomassie Brilliant Blue dye. In most experiments, 10 μ l of experimental sample was loaded per gel lane. Because of variability in protein content between nuclear preparations (see above) this represented between 40 and 80 μ g of total nuclear protein. It is important to note that equal volumes of sample were loaded in any individual experiment, so that direct comparisons of staining intensities could be made.

Protein Blotting and Immunostaining—Samples of nuclei and calpain were electrophoresed as described above, electroblotted onto nitrocellulose, and immunostained for calpain small subunit as previously described (18). A monoclonal antibody, P-1, which recognizes bovine calpain small subunit was used as the first antibody. Alkaline phosphatase-coupled second antibody was utilized, and the nitro blue tetrazolium, indolylphosphate staining procedure was employed (19).

Proteolysis of Nuclear Proteins by Calpains—Samples of nuclei were washed several times by centrifugation and resuspension in the original sample volume of buffer A containing various concentrations of Ca^{2+} . Except where otherwise indicated, they were incubated at 25 °C with 30 μ g *m*-calpain/ml (a calpain to nuclear protein ratio of approximately 1:200) for 20 min. One-half volume of SDS sample buffer was added to the samples, and they were heated to 100 °C for 5 min. Twenty- μ l aliquots were subjected to SDS-polyacrylamide gel electrophoresis. For quantitating proteolysis of the nuclear proteins, the Coomassie-stained gels were scanned with a laser densitometer. Peak areas of protein bands at 120–140 kDa in treated samples were compared to peak areas in a sample of untreated nuclei applied to the same gel.

Calpain Assay—Calpain activity was routinely measured by release of ^{14}C -labeled trichloroacetic acid-soluble peptides from [^{14}C]methyl casein as previously described (20). In some cases the spectrophotometric assay of Dayton *et al.* (8) was used. A unit of calpain activity in the latter assay is defined as the amount of enzyme which produces sufficient trichloroacetic acid-soluble casein peptides to increase optical density at 278 nm by 1.0 absorbance unit.

Treatment of Nuclei with Nucleases and Phospholipase C—Nuclei were incubated for 60 min in an ice bath with 0.25 mg of DNase/ml, 0.25 mg of RNase/ml, 0.25 mg of each nuclease/ml, or 10 μ g of phospholipase C/ml. The samples were then washed three times by centrifugation for 5 min at 12,000 $\times g$ and resuspension in buffer A containing 200 μM Ca^{2+} . The final resuspended samples were incubated with *m*-calpain as described above.

Reconstitution of *m*-Calpain Activation with DNA—Samples of nuclei were treated with nucleases (DNase and RNase, 0.1 mg/ml each), and washed three times in buffer A (without Ca^{2+}) as described in the preceding section. The nuclear pellets were resuspended in buffer A containing 0.6 M NaCl to solubilize the internal nuclear matrix. To one-half of the sample, DNA was added to 2 mg/ml. Except where indicated salmon testis DNA was utilized. 0.5-ml aliquots of the DNA-containing sample, and the control sample without added DNA, were dialyzed for 2 h against 250 ml of Ca^{2+} -*N*-hydroxyethylthylenediamine-*N,N,N'*-triacetic acid buffers containing various concentrations of free Ca^{2+} . Aliquots of the final dialyzed samples were then incubated with *m*-calpain as described above. Twenty-five- μ l samples were subjected to SDS-polyacrylamide gel electrophoresis.

RESULTS

Copurification of Ca^{2+} -dependent Proteolysis of Nuclear Proteins and Caseinolytic Activity of *m*-Calpain—As indicated under "Experimental Procedures," *m*-calpain purified by an established protocol (10) did not contain detectable μ -calpain activity. However, one other calpain isozyme, p94, has been discovered (21), and it is possible that more isozymes will be discovered in the future. Therefore, it was possible that an unidentified Ca^{2+} -dependent and calpastatin-sensitive protease was present as a contaminant in the *m*-calpain preparation and was responsible for nuclear protein proteolysis at low Ca^{2+} concentrations.

To determine whether this was the case, purification of an *m*-calpain preparation was monitored using the standard caseinolytic assay, as well as incubation with nuclei to detect

proteolysis of nuclear proteins as described under "Experimental Procedures." There was no detectable separation of the two activities on Bio-Gel A-1.5m chromatography (Fig. 1), DEAE-Sepharose chromatography developed with a NaCl gradient (Fig. 2), or the final phenyl-Sepharose chromatography step of the purification protocol (Fig. 3). These results indicate that the proteolysis of nuclear proteins at low Ca^{2+} concentrations is catalyzed by *m*-calpain.

Effect of DNase, RNase, and Phospholipase C Treatment of Nuclei on *m*-Calpain Calcium Requirement—Nuclei were preincubated with nucleases or phospholipase C as described under "Experimental Procedures," and then incubated with *m*-calpain for 20 min in the presence of 200 μM Ca^{2+} . Neither RNase nor phospholipase C had any influence on the degree of proteolysis (Fig. 4, lanes 4 and 6). However, pretreatment with DNase prevented cleavage of the high molecular weight substrates (Fig. 4, lanes 3 and 5). There was some decrease in histone H1 content after DNase preincubation in the experiment shown in Fig. 4. This was not dependent upon the presence of calpain, and could be substantially reduced by addition of phenylmethylsulfonyl fluoride to the DNase immediately prior to addition to the nuclei and by using lower concentrations of DNase in the incubation (for example, see

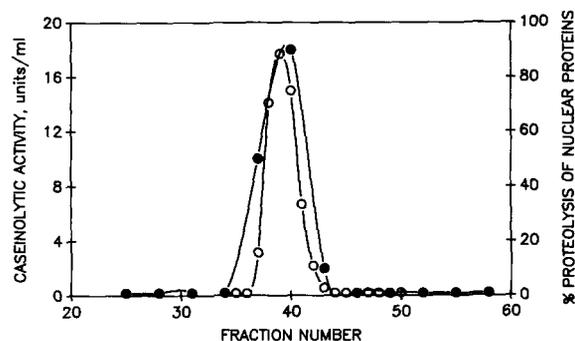


FIG. 1. Coelution of *m*-calpain caseinolytic activity and nuclear protein proteolyzing activity on Bio-Gel A-1.5m chromatography. One kg of beef heart was homogenized and centrifuged, and the supernatant was subjected to batch DEAE-Sepharose chromatography as described previously (10). The pooled calpain fractions from DEAE-Sepharose (25 ml) were applied to a 2.6 \times 120-cm column of Bio-Gel. Ten-ml fractions were collected and assayed for caseinolytic activity by the spectrophotometric assay (○) or proteolysis of high molecular weight nuclear proteins in the presence of 200 μM Ca^{2+} (●) as described under "Experimental Procedures."

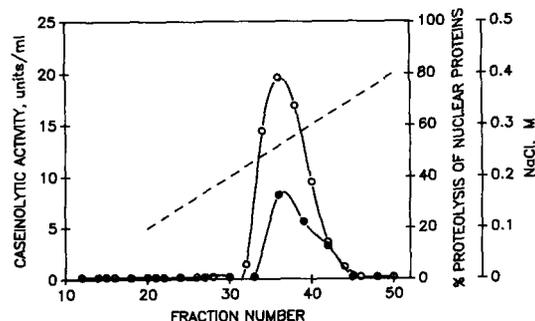


FIG. 2. Coelution of *m*-calpain caseinolytic activity and nuclear protein proteolyzing activity on DEAE-Sepharose chromatography. Ten ml of pooled fractions 38 to 41 from the Bio-Gel chromatography step in Fig. 1 were applied to a 10-ml column of DEAE-Sepharose, and eluted with a 50-ml 0.8 M to 5 M NaCl gradient in 50 mM imidazole-HCl, 0.2 mM EGTA, 1 mM DTT, pH 7.4. One-ml fractions were collected, and 10- μ l samples were assayed for caseinolytic activity (○) or activity on nuclear proteins in the presence of 200 μM Ca^{2+} (●).

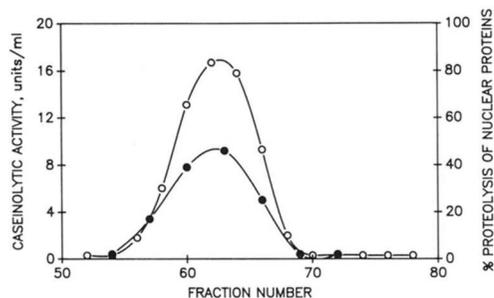


FIG. 3. Coelution of *m*-calpain caseinolytic activity and nuclear protein proteolytic activity on phenyl-Sepharose. Fractions 34 to 40 from DEAE-Sepharose chromatography (Fig. 2) were pooled and applied to a 10-ml column of phenyl-Sepharose equilibrated in 50 mM imidazole-HCl, 0.2 mM EGTA, 250 mM NaCl, 1 mM DTT, pH 7.4. The column was developed with a 50-ml linear gradient using equilibration buffer as the start buffer, and 20 mM Tris-HCl, 1 mM EGTA, 1 mM DTT, pH 7.5, as the end buffer. The column was further washed with 50 ml of end buffer. One-ml fractions were collected and assayed for proteolytic activities as in Figs. 1 and 2.

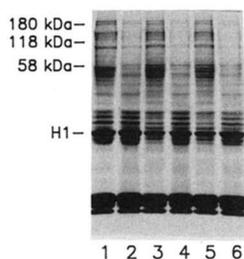


FIG. 4. Effect of nuclease or phospholipase C treatment on the susceptibility of nuclear matrix proteins to cleavage by *m*-calpain. Samples of nuclei were preincubated in storage buffer with nucleases or phospholipase C, washed, and then incubated with *m*-calpain. Details are given under "Experimental Procedures." Lane 1, minus calpain control; lane 2, no preincubation; lane 3, nuclei preincubated with DNase; lane 4, nuclei preincubated with RNase; lane 5, nuclei preincubated with both DNase and RNase; lane 6, nuclei preincubated with phospholipase C. Protein standards applied to a separate lane were α -macroglobulin (180 kDa), β -galactosidase (118 kDa), and pyruvate kinase (58 kDa). The endogenous nuclear protein histone H1 is also marked.

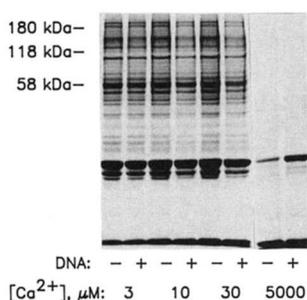


FIG. 5. Addition of DNA to DNase-treated nuclei restored sensitivity of matrix proteins to cleavage by *m*-calpain at low $[Ca^{2+}]$. DNase-treated nuclei were preincubated with salmon testis DNA at different Ca^{2+} concentrations and incubated with *m*-calpain as described under "Experimental Procedures." A control sample of DNase-treated nuclei was subjected to the same preincubation and calpain incubation procedure, but without added DNA.

Fig. 5). Hence, the histone H1 protease appeared to be present in the DNase preparation. In the many studies we have done so far, the effect of DNase pretreatment on calpain-dependent proteolysis has not correlated with the degree of histone H1 loss produced by the contaminating protease.

Reversal of the DNase Effect by the Addition of DNA—Nuclei were incubated with DNase, washed, solubilized with

0.6 M NaCl, and dialyzed in the presence or absence of 2 mg of salmon testis DNA/ml as described under "Experimental Procedures." After dialysis, the samples were incubated in the presence of *m*-calpain, and the degree of proteolysis of the nuclear proteins was assessed (Fig. 5). The results indicated that the addition of exogenous DNA could restore the sensitivity of the nuclear substrates to *m*-calpain cleavage. Significant proteolysis of the high molecular mass nuclear proteins was detectable at $3 \mu M Ca^{2+}$ when DNA was present: comparison of densitometric scans of Fig. 5, lanes 1 and 2, revealed a 20% decrease in staining intensity of the 120–140-kDa nuclear proteins in the sample incubated with calpain and DNA. Further proteolysis was noted in the DNA-containing samples at 10 and $30 \mu M Ca^{2+}$. The substantial proteolysis observed at Ca^{2+} concentrations between 3 and $30 \mu M$ was in accord with the previously documented Ca^{2+} requirement for *m*-calpain-catalyzed cleavage of nuclear proteins in the presence of endogenous DNA (10). At $5 mM Ca^{2+}$, the nuclear proteins were proteolyzed whether DNA was present or not; therefore, the effect of DNA was to decrease the Ca^{2+} concentration needed to activate *m*-calpain.

Salmon testis DNA, calf thymus DNA, and *E. coli* DNA activated *m*-calpain at low Ca^{2+} (Fig. 6). However, as expected from the results presented in Fig. 4, yeast RNA was a much less effective activator (Fig. 6). The synthetic polydeoxyribonucleotides, polydeoxyadenylate, polythymidylate, and polydeoxyadenylate:polythymidylate copolymer were also effective activators of *m*-calpain (Fig. 6). Prokaryotic (*E. coli*) DNA and eukaryotic (calf thymus) DNA were equally effective in increasing *m*-calpain activity (Fig. 7). Relatively high concentrations of DNA were required to reconstitute calpain activity. However, this was consistent with the amount of DNA removed from the nuclei by DNase treatment: in two different nuclear samples, 1.31 and 1.43 mg of DNA was solubilized per ml of nuclei, as determined by the diphenylamine assay (15).

Investigation of the Interaction of *m*-Calpain with DNA—To further explore the mechanism for activation of *m*-calpain by DNA, the activity of *m*-calpain on ^{14}C -methylated casein was measured in the absence or presence of 2 mg of salmon testis DNA/ml. *m*-Calpain had virtually no detectable caseinolytic activity at $200 \mu M Ca^{2+}$ either in the presence or absence of DNA. In another experiment, $8 \mu g$ of *m*-calpain was incubated with 30 mg of DNA-cellulose gel equilibrated in 100 μl of 100 mM MOPS, 1 mM $MgCl_2$, 50 mM NaCl, 1 mM

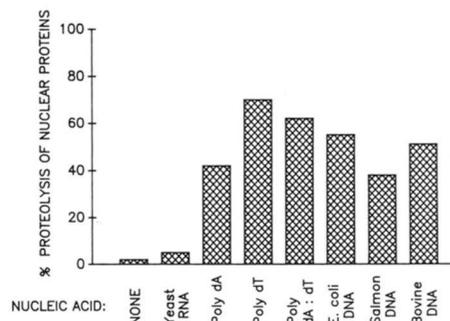


FIG. 6. Reconstitution of nuclear protein sensitivity to *m*-calpain cleavage by addition of polydeoxyribonucleotides. Nuclei were treated with nucleases, washed, and reconstituted with various nucleic acids as described under "Experimental Procedures." Samples were incubated with $20 \mu g$ of *m*-calpain/ml in the presence of $200 \mu M Ca^{2+}$ for 20 min, electrophoresed, and Coomassie-stained as described under "Experimental Procedures." The gel lanes were then scanned in a densitometer, and the proteolysis of the 100–200-kDa proteins was estimated by loss of staining intensity relative to untreated nuclei.

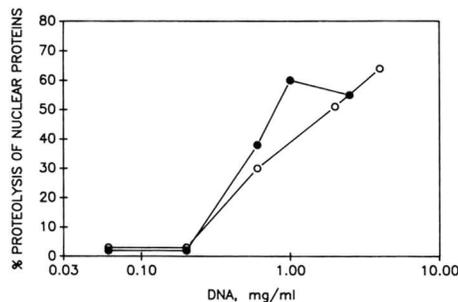


FIG. 7. Concentration dependence of the activation of *m*-calpain by *E. coli* or calf thymus DNA. Nuclease-treated nuclei were reconstituted as described under "Experimental Procedures" with the indicated concentration of *E. coli* (●) or calf thymus (○) DNA. Samples were incubated with 20 μ g of *m*-calpain/ml in the presence of 200 μ M Ca^{2+} , electrophoresed, stained and scanned as described in Fig. 6.

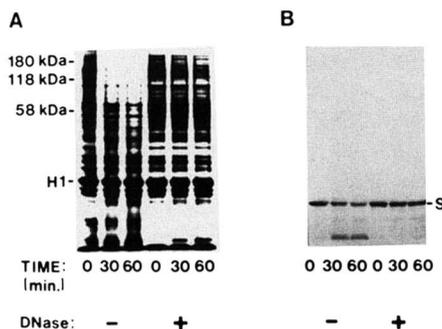


FIG. 8. Autoproteolysis of *m*-calpain during proteolysis of nuclear proteins. Nuclei or DNase-treated nuclei were incubated in the presence of 200 μ M Ca^{2+} with 30 μ g of *m*-calpain/ml for various times and subjected to gel electrophoresis, and the gels were stained for protein with Coomassie Blue (A) or blotted and immunostained for calpain small subunit (B) as described under "Experimental Procedures." Molecular weight markers are indicated on the Coomassie-stained gel. In B, the position of the calpain small subunit is marked with an S.

DTT, and 100 μ M $Ca(OAc)_2$, pH 7.0. After 30 min of incubation at 25 $^{\circ}$ C with continuous mixing, the mixture was centrifuged. Virtually all of the calpain activity was recovered in the gel supernatant. Furthermore, the recovered calpain was inactive at 200 μ M Ca^{2+} in the standard 14 C-caseinolytic assay. In similar experiments, *m*-calpain was preincubated with DNA and 200 μ M Ca^{2+} , followed by removal of the DNA by DNase I. After this treatment, *m*-calpain was not effective in proteolysis of proteins in DNase-treated nuclei in the presence of 200 μ M Ca^{2+} . Thus, DNA did not directly induce autoproteolytic activation of *m*-calpain.

In contrast with the above findings, when *m*-calpain was incubated with intact nuclei, there was clear evidence of autoproteolysis of the small subunit during breakdown of nuclear proteins (Fig. 8). In DNase-treated nuclei, no autoproteolysis was evident.

DISCUSSION

Previous studies have indicated a possible function for *m*-calpain in the turnover of nuclear proteins. Tokes and Clawson (22) demonstrated the presence of a Ca^{2+} -activated nuclear protease in rat liver nuclear scaffold preparations. The content of this protease in nuclei was increased following *in vivo* administration of the hepatocarcinogen thioacetamide (22). Microinjection of *m*-calpain reportedly accelerated mitosis in PtK₁ cells (3), and microinjection of calpastatin could arrest mitosis under some conditions. It was previously shown

that *m*-calpain could proteolyze a number of high molecular weight proteins in rat liver nuclei at Ca^{2+} concentrations as low as 3 μ M (10).

The decreased Ca^{2+} requirement for *m*-calpain activity on nuclear substrates depended on the presence of DNase-sensitive DNA in the nuclei (Fig. 4). Phospholipids have been shown to decrease the Ca^{2+} requirement of *m*-calpain to the 10^{-4} M range (23, 24). However, phospholipase C treatment of nuclei did not influence the Ca^{2+} sensitivity of *m*-calpain (Fig. 4). The lack of influence of RNase treatment (Fig. 4) indicated that RNA was not involved in the nuclear activation of *m*-calpain.

The reconstitution studies unequivocally demonstrated the requirement for DNA in activating *m*-calpain. RNA did not appear to be capable of effectively reconstituting calpain sensitivity to the nuclear proteins (Fig. 6). In a few of the other experiments we have performed, there was some increased proteolysis of the nuclear proteins by *m*-calpain in the presence of RNA. The greatest stimulation of nuclear protein degradation by RNA that we have observed thus far was 12% proteolysis, under the same experimental conditions as presented in Fig. 6. It was therefore apparent that RNA was less effective than DNA in activating *m*-calpain. The source of DNA used to activate calpain was unimportant; *i.e.* prokaryotic DNA was as effective as eukaryotic DNA (Fig. 7). This implies that specific DNA binding sites for eukaryotic DNA binding proteins are not essential for the activation phenomenon. Indeed, the synthetic single stranded homopolymers, polythymidylate and polydeoxyadenylate, could activate calpain (Fig. 6).

DNA did not appear to activate *m*-calpain by directly binding to it, since *m*-calpain did not bind to DNA-cellulose, and its caseinolytic activity was not influenced by DNA. The latter observation does not appear to be uniquely associated with calpain action on casein, since preliminary experiments indicate that the proteolysis of other substrates by *m*-calpain, including several partially purified nuclear matrix proteins, is not influenced by DNA (data not shown). DNA did not appear to allow autoproteolytic activation of *m*-calpain in the presence of low Ca^{2+} concentrations. The latter is a proposed mechanism for activation of calpains by acidic phospholipids (25).

While little is presently known about the functions and properties of individual nuclear matrix proteins, there is recent evidence that a number of these proteins, including several having molecular masses greater than 100 kDa, are capable of binding DNA in blot overlay analyses (26). Based on these observations and our studies, it seems reasonable to propose that DNA interacts in a complex manner with calpain and high molecular mass proteins in the intact nuclear matrix. Autoproteolysis of *m*-calpain appeared to require the presence of the nuclear protein substrates as well as DNA. This observation is consistent with a previous report that the presence of digestible proteins allows autoproteolytic activation of erythrocyte μ -calpain at decreased Ca^{2+} concentrations (27).

The results of our studies indicate that DNA plays an important role in modulating the activity of *m*-calpain on nuclear matrix proteins. Further investigation is required to determine the nature of the interactions within the nuclear matrix that allow DNA to activate *m*-calpain, and to determine whether DNA interactions are important in regulating the proteolysis of other DNA-binding proteins by *m*-calpain. Of particular interest is the proteolysis of short-lived DNA-binding proteins, *e.g.* the transcription factors *c-fos* and *c-jun*. Recent studies indicate that these two proteins are exquisitely sensitive to calpain cleavage *in vitro* and that *c-jun* is a

substrate for calpains *in vivo* (28).

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