J. Biochem. 122, 865-871 (1997)

Identification of Endogenous Substrates for *Drosophila* Calpain from a Salt-Extracted Fraction of *Drosophila* Ovaries[1]

Susumu Amano,*‡ Hiroshi Kawasaki,† Shoichi Ishiura,* Seiichi Kawashima,† Koichi Suzuki,* and Yasufumi Emori‡,2

*Laboratory of Molecular Structure and Function, Department of Molecular Biology, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113; the †Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, 3-8-22 Honkomagome, Bunkyo-ku, Tokyo 113; and the ‡Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

Received for publication, June 20, 1997

Drosophila **calpain (Dm-calpain) produced in *Escherichia coli* has a distinct Ca^{2+}-dependent activity. By using a recombinant Dm-calpain, we searched for its substrates occurring in *Drosophila* ovaries, where Dm-calpain is expressed. Among a number of major proteins, several proteins in a salt-extracted fraction were selectively degraded by Dm-calpain in a Ca^{2+}-dependent manner. The major substrates were identified by microsequencing the lysylendopeptidase-digested proteins. Three ribosomal proteins, the L5, L7, and L8 subunits of the 60S ribosome, were found to be potential Dm-calpain substrates. In addition, the α subunit of elongation factor-1 (EF-1α), a multi-functional protein involved in both protein synthesis and cytoskeletal regulation, was shown to be cleaved by Dm-calpain into several distinct fragments when expressed as a GST-fusion protein. Endogenous EF-1α in ovary extracts was also shown by western blot analysis to be similarly degraded. These observations suggest that Dm-calpain may regulate protein synthesis and cytoskeletal structure through its degradative or processing activity.**

Key words: bacterial-expression, calpain, *Drosophila*, EF-1α, limited-proteolysis.

Calpain [EC 3.4.22.17], an intracellular calcium-dependent cysteine protease ubiquitously distributed in animal tissues, is thought to perform various physiological functions as a biomodulator by cleaving substrate proteins to a limited extent, and altering or down-regulating their activities or functions (*1, 2*). *In vitro* calpain substrates include various types of proteins such as transcription factors (*3, 4*), calmodulin-binding proteins (*5, 6*), components of receptor-mediated signal transduction (*7*), and cytoskeletal proteins (*8-10*). However, the consequence of cleaving these substrates *in vivo* is not clear in terms of intracellular signaling, and hence, the definite physiological functions of calpain remain unclear.

On the other hand, the structural and biochemical features of calpains have been well defined by molecular analysis. In mammals, two ubiquitously distributed calpain species showing different calcium requirements, termed μ- and *m*-calpains, have been characterized (*11-14*), and two tissue-specific calpains, termed nCL-1 (p94) and nCL-2, have been identified in skeletal muscle and stomach, respectively (*15-17*). These calpains, which occur commonly in mammals, are similar but distinct in their amino acid sequences and can be structurally divided into four domains, of which the second and fourth domains (domains II and IV) are assigned as the catalytic domain and the calcium-binding domain, respectively (*11-14*).

Recently, novel calpain-like molecules have been identified in invertebrates such as *Schistosoma mansoni* (*18*), *Caenorhabditis elegance* (*19-21*), and *Drosophila melanogaster* (*22-24*). Moreover, the *PalB* gene product of *Aspergillus nidulans*, a filamentous fungus, shows similarities in amino acid sequence to domain II of mammalian calpain and has been shown to be involved in the signaling of ambient pH (*25*). These invertebrate calpains can be structurally classified into typical and atypical types. Typical calpains, including Dm-calpain (*Calp A* protein) in *D. melanogaster* and Sm-calpain in *S. mansoni*, are quite similar in domain structure to mammalian ubiquitous μ- and *m*-calpains. Atypical calpains, including *palB* protein from *A. nidulans*, *C. elegance* calpains such as *Tra3* protein, and *sol* protein from *D. melanogaster*, are significantly different from mammalian calpains in their domain structure: they contain a protease domain similar to those of typical calpains, but lack one to three of the four domains contained in typical calpains, and in some cases, like *Drosophila sol*, additional domains such as Zn-finger motifs are included (*22*). Since studies in invertebrates have advantages over studies in vertebrates in that developmental and genetic analyses can be applied, invertebrates may provide better clues to clarify the physiological functions of calpain.

We have shown that Dm-calpain might be involved in early embryogenesis, especially in the organization of the actin-related cytoskeleton (*23*). In terms of its structural features, Dm-calpain is the most related to mammal

[1] This work was supported by Grants-in-Aid for Scientific Research (B) and on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Phone: +81-3-3812-2111 (Ext. 4406), Fax: +81-3-5684-2394

calpains among invertebrate calpains; thus, Dm-calpain appears most suitable for the above studies. However, the biochemical characteristics of Dm-calpain, especially those concerning its endogenous substrates, have not yet been studied.

In this study, we constructed an *Escherichia coli* expression system for Dm-calpain in an active form. Using recombinant Dm-calpain, we investigated and identified several Dm-calpain substrates including three ribosomal proteins from a salt-extracted fraction of *Drosophila* ovaries. The α subunit of translational elongation factor-1 (EF-1α) was shown to be degraded by Dm-calpain in a limited manner, a typical proteolytic mode of mammalian calpains. These results indicate that Dm-calpain is comparable to vertebrate calpains in its biochemical nature as well as structural features.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—For the construction of a Dm-calpain expression plasmid, the total coding region of Dm-calpain cDNA (GenBank™/EMBL, X78555; Ref. *23*) was amplified by high-fidelity thermostable DNA polymerase (*Pfu* DNA polymerase, Stratagene) using a pair of primers which had the sequences AGGAATTCGGACCGACTTG-AGGGGATTC (including the putative initiation codon changed to a TCG codon (double-lined), where an artificial *Eco*RI site is underlined) and TTGAATTCGTATGTGGT-GAAGCAG (corresponding to the antisense strand of the 3′-noncoding sequence, where an artificial *Eco*RI site is underlined). The amplified DNA was digested with *Eco*RI and inserted into the *Eco*RI site of pUC118. Plasmids harboring the Dm-calpain cDNA in the desired direction (*i.e.* the same orientation as the *lacZ* promoter) were selected.

The putative coding region of EF-1α of *D. melanogaster*, characterized previously (GenBank™/EMBL, X06869; Ref. *26*), was amplified using the larval cDNA and a pair of primers, TACCCGGGCATGGGCAAGGAAAAG, corresponding to the initiation site, where the underlined and double-lined sequences are an artificial *Sma*I site and the initiation site, respectively, and TAGCGGCCGCTACTTC-TTGCCCTTGGT, corresponding to the antisense strand of the 3′-noncoding region, where the underlined sequence is an artificial *Not*I site. An amplified DNA band with the expected length (1.6 kbp) was subcloned after *Sma*I/*Not*I digestion into GST-fusion expression vector pGEX-5X-1 (Pharmacia Biotech).

Bacterial Expression of Dm-Calpain and EF-1α—E. coli AD202 (*27*) carrying the Dm-calpain expression plasmid constructed above was plated into LB/agar containing ampicillin (100 μg/ml). Colonies were picked up and inoculated in LB medium containing ampicillin (100 μg/ml) and grown at 32°C to $A_{550}=1.6$. Production of the recombinant protein was induced by the addition of IPTG at a final concentration of 1 mM. The culture was further incubated for 2 h at 32°C, and the cells were harvested by centrifugation, washed once in buffer I (20 mM K-Hepes, pH 7.5, 1 mM EDTA, 0.1 mM DTT), and suspended in buffer I. All subsequent steps were carried out on ice. The cells were disrupted by sonication, and the crude extract was obtained by centrifugation at 15,000×g for 10 min. One-ninth volume of 5 M NaCl solution was added to the extract to give a final concentration of 0.5 M, and the mixture was applied to a column of phenyl-Sepharose (Pharmacia Biotech). After washing with buffer I containing 0.5 M NaCl, bound materials were eluted with buffer I. Fractions containing calpain activity measured as described below were collected and used for further studies. The fractions were completely devoid of any proteolytic activities other than Dm-calpain expressed under the conditions of the calpain assay.

The GST-fusion EF-1α constructed above was expressed essentially in the same way as Dm-calpain except for an IPTG induction period (1 h). The GST-fusion EF-1α was purified from the crude extract of the cells producing recombinant proteins by glutathione-Sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech).

Extraction of Drosophila Ovaries and Protein Fractionation—Fly stocks of *D. melanogaster* Canton-S were maintained under standard culture conditions (*28*). Ovaries were dissected from adult ethyl ether-anesthetized female, and homogenized in three volumes of buffer I containing 20 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride at 0°C with 20 strokes of a Teflon homogenizer. The homogenate was centrifuged at 12,000×g for 10 min at 4°C, and the supernatant was collected and used as the soluble fraction. Three volumes of buffer I containing 0.5 M NaCl were added to the pellet, which was homogenized again in the same way as in the previous step. The supernatant obtained was used as the salt-extracted fraction.

Calpain Reaction—Calpain activity was measured in 20 μl (final volume) of reaction mixture containing 20 mM K-Hepes, pH 7.5, 0.5 mM DTT, 2 μg of casein, 7 mM EDTA or $CaCl_2$, and 5 μl of enzyme fraction. The reaction mixtures were incubated at 30°C for 30 min, and the reaction was terminated by adding an equal volume of 2× SDS sample buffer and boiling for 10 min. Calpain reactions using ovary extracts and GST-fusion EF-1α as substrates were carried out essentially under the same conditions as the above casein assay, with the cysteine protease inhibitor E64 sometimes added as a control assay. The final reaction mixtures were electrophoresed on SDS-polyacrylamide gels (*29*) stained with Coomassie Brilliant Blue R250 or subjected to silver staining using a 2D-silver stain kit (Daiichi Pure Chemicals).

Identification of Calpain Substrates Recovered from SDS-Polyacrylamide Gel—Protein bands in the SDS-polyacrylamide gels which were identified as Dm-calpain substrates and stained with Coomassie Brilliant Blue R250 were excised. The gel fragments were washed with 50% acetonitrile in 0.2 M ammonium carbonate, pH 8.9, and digested with lysylendopeptidase (Wako Pure Chemicals; 33 μg/ml) diluted with 0.02% Tween 20 in 0.2 M ammonium carbonate, pH 8.9, at 37°C for 24 h. The following protocol is a slight modification of the original procedure described by Kawasaki *et al.* (*30*). After digestion, the peptides were extracted from the gel-matrix and separated by reversed-phase HPLC using a C18 column (3.9×150 mm; Waters) equilibrated in 98% buffer A [0.1% TFA (v/v)]/2% buffer B (0.1% TFA in 100% acetonitrile). Peptide separation was carried out by elution with an acetonitrile gradient of 98% buffer A/2% buffer B to 100% buffer B at a flow rate of 0.5 ml/min for 60 min using a Waters 600E multisolvent delivery system. Each of the separated pep-

tides was collected and subjected to sequencing analysis using an automatic protein sequencer (Applied Biosystems; Model 492).

Western Blot Analysis—SDS-polyacrylamide gel electrophoresis was performed using the buffer system described by Laemmli (*29*) and immunoblots on PVDF membranes (polyvinylidene difluoride; Millipore) were performed by a standard protocol as described previously (*31*). The polyclonal antibody against yeast EF-1α was generously supplied by Prof. Masazumi Miyazaki (Nagoya University).

Immunostaining of Drosophila Ovaries—Ovaries were dissected from *D. melanogaster* Canton-S and processed for immunostaining using anti-Dm-calpain antibody as described previously (*23, 32*). Briefly, the ovaries were fixed with 4% paraformaldehyde in PBS containing 0.1% Triton, washed in PBS containing 0.1% Triton, and incubated with the primary antibody (anti-Dm-calpain antibody). After washing with the same buffer, an anti-rabbit IgG conjugated with FITC (fluorescein isothiocyanate) was added, and the samples were incubated for 2 h and washed. Signals were observed under a confocal microscope (Olympus, LSM-GB200).

RESULTS

Functional Expression of Dm-Calpain in E. coli—We tried to express Dm-calpain as an active enzyme using several expression vectors. Most approaches, including the GST-fusion method, failed because Dm-calpain was recovered in insoluble fractions (inclusion bodies) and showed no proteolytic activity either with or without Ca^{2+}. When, we selected a relatively weak promoter (*lacZ* of pUC vectors), low incubation temperature (30-32°C), and a specific *E. coli* strain AD202 (*27*) for the production of active Dm-calpain, however, under the conditions detail in "EXPERIMENTAL PROCEDURES." about half the expressed Dm-calpain was recovered in the soluble fraction. Since significant amounts of Ca^{2+}-dependent proteolytic activity were detected in extracts of *E. coli* harboring the expression plasmid and not in extracts of control *E. coli* harboring the plasmid without the cDNA insert (data not shown), recombinant Dm-calpain was partially purified by phenyl-Sepharose column chromatography (Fig. 1A). Although the obtained fraction contained several *E. coli* proteins in addition to Dm-calpain, it was completely devoid of neutral protease activity other than the calcium-dependent activity of Dm-calpain. The recombinant Dm-calpain thus obtained degraded casein as a substrate in a calcium-dependent manner (Fig. 1B) when assayed under the conditions described in "EXPERIMENTAL PROCEDURES." The calcium requirement for enzyme activity was around 5 mM, which

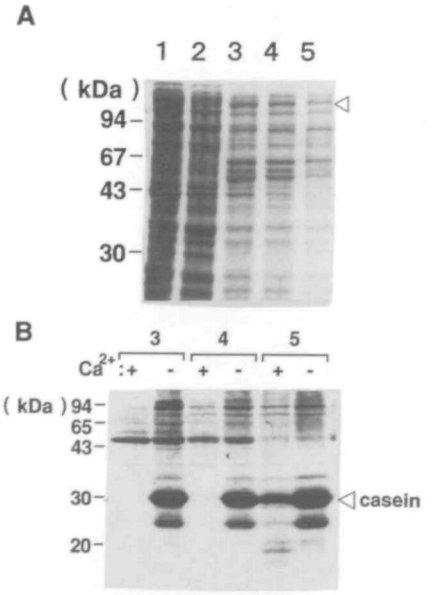

Fig. 1. **Expression of Dm-calpain in *E. coli* and the measurement of its activity.** A: Partial purification of recombinant calpain. Lane 1, crude *E. coli* extract; lane 2, flow-through fraction of the phenyl-Sepharose column; lanes 3, 4, and 5, eluates from the phenyl-Sepharose column. The open triangle denotes Dm-calpain expressed in *E. coli*. B: Calpain assay of partially purified recombinant Dm-calpain. Enzyme fractions of the eluates shown by the same numbers as A were incubated with casein in the presence (+) or absence (−) of Ca^{2+} under the conditions described in "EXPERIMENTAL PROCEDURES." Each gel was stained with Coomassie Brilliant Blue R250.

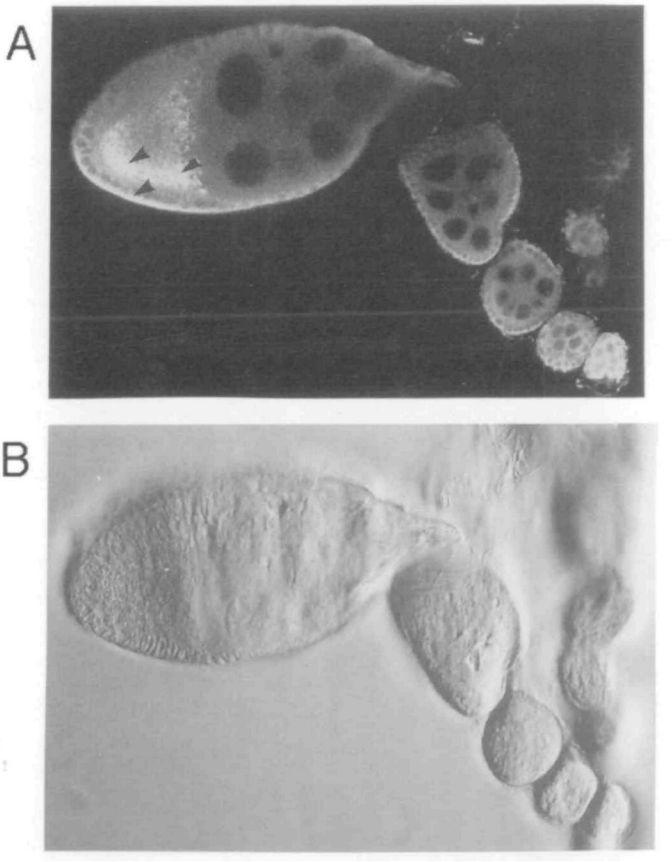

Fig. 2. **Distribution of Dm-Calpain in *Drosophila* ovary.** Egg chambers are dissected and fixed as described under "EXPERIMENTAL PROCEDURES." A: Immunostaining of *Drosophila* egg chambers using anti-Dm-calpain antibody was visualized by FITC-conjugated secondary antibody. Fluorescence signals were observed under a confocal microscopy. B: Transparent view of the same sample as in A, which visualizes the cellular structure. Positive signals are widely distributed in the cytosol of the oocyte, and concentrated dots under the cell membrane were observed in oocyte and follicle cells (indicated by arrowheads). In eggs chambers in the early stage, strong signals are broadly spread in the cytosol of the nurse cells.

is roughly the same as native *Drosophila* calpain (*33*), probably corresponding to our Dm-calpain.

Dm-Calpain Degraded Several Proteins in Drosophila Ovaries in a Ca^{2+}-Dependent Manner—To identify endogenous substrates for Dm-calpain, we first used total extracts prepared from adult flies. However, we could detect no protein bands that were specifically degraded by Dm-calpain on SDS-polyacrylamide gels, probably due to the complexity of the proteins contained in the extract. We then chose the ovary as a source material, because the ovary contains a limited number of cell types including germline cells (nurse cells and oocyte) and somatic cells (follicle cells and muscle cells), and because a significant amount of Dm-calpain occurs in the ovary as described below. As shown in Fig. 2, Dm-calpain is broadly distributed in the cytosol of the oocyte, nurse cells, and follicle cells, and, in addition, strong signals are observed in the surface regions of the oocyte and follicle cells. We then fractionated ovary proteins into a soluble fraction containing mainly cytosolic proteins and a salt-extracted fraction containing cytoskeletal proteins, their associated proteins, and membrane-associated proteins. Each fraction was incubated with recombinant Dm-calpain in the presence of calcium ions (Fig. 3) and the profiles were compared with those obtained in the presence of E64, a calpain inhibitor. When the soluble fraction was used, no clear difference was observed between the SDS-PAGE profiles in the presence and absence of E64 (lanes 1 and 2), although the intensities of several bands seemed to change. This negative result might be due to the complexity of this fraction, *i.e.*, the overlap of plural proteins showing the same electrophoretic mobilities. It would not be due either to the absence of calpain substrates or to the endogenous inhibitory activity against Dm-calpain, because at least some of the calpain substrates described below are contained in the soluble fraction, and because Dm-calpain is capable of cleaving its substrates, such as casein, which were added exogenously to reaction mixtures containing the soluble fraction. When the salt-extracted fractions were used, Dm-calpain degraded several proteins selectively and Ca^{2+}-dependently as shown in Fig. 3 (indicated by the open triangles). It should be noted that Dm-calpain degraded only a small number of major proteins among the numerous protein bands visualized on the SDS-polyacrylamide gel, showing Dm-calpain to be a "selective" enzyme. Since this degradation was dependent on Ca^{2+} and inhibited by E64, and since this cleavage did not occur in the absence of exogenously added Dm-calpain (lane 5, Fig. 3), the bands in lane 3 of Fig. 3 can be regarded as true Dm-calpain substrates. Endogenous Dm-calpain activities as well as other proteolytic activity, if they occurred, were negligible.

Identification of Dm-Calpain Substrates in the Ovary—To identify the protein species degraded by Dm-calpain as described above, each of the protein bands was excised from the gel and digested with lysylendopeptidase. The peptide fragments generated were separated on reversed-phase HPLC (Fig. 4), and several peptides (indicated in the

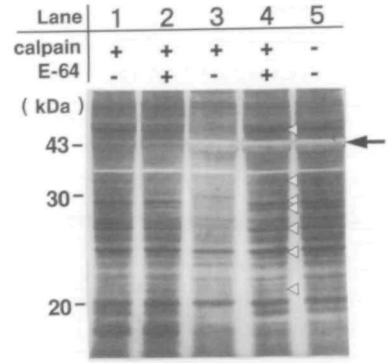

Fig. 3. **Degradation of endogenous proteins in ovary by Dm-calpain.** Soluble fractions (lanes 1 and 2) and salt-extracted fractions (lanes 3, 4, and 5) were incubated with Dm-calpain (Fig. 1) in the presence or absence of E64, electrophoresed on 10% SDS-polyacrylamide gels, and stained with a silver staining kit. Cytosolic actin contained in the salt-extracted fraction is indicated by an arrow. Lane 5 denotes a sample incubated in the absence of the Dm-calpain fraction. Protein bands subjected to Ca^{2+}-dependent degradation by Dm-calpain are indicated by open triangles.

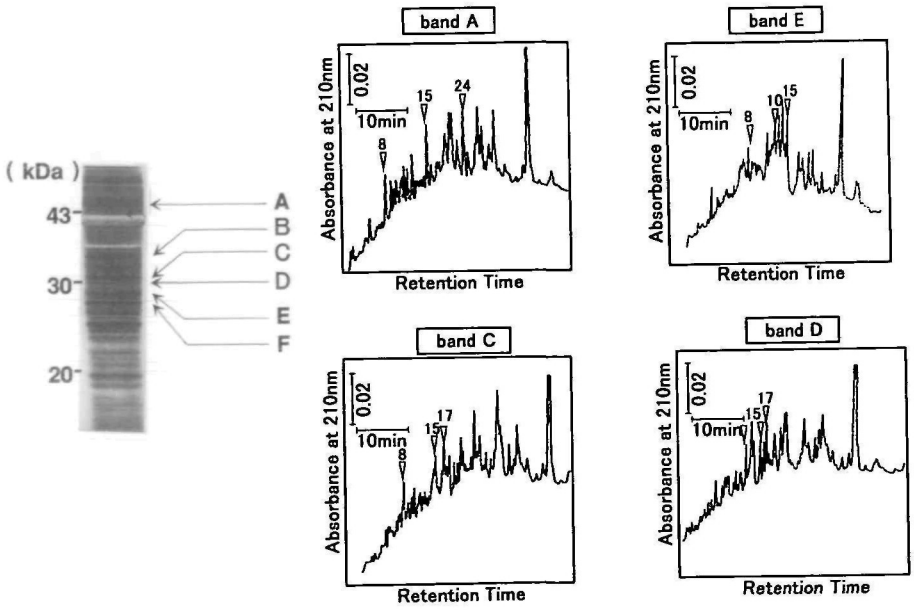

Fig. 4. **Separation of peptides on reversed-phase HPLC.** The left panel denotes the SDS-PAGE profile; bands (A-F) were excised. Each of the excised bands was digested with lysylendopeptidase, and the resulting peptides were separated on reversed-phase HPLC under the conditions described in "EXPERIMENTAL PROCEDURES," as shown in the four right panels. From each reversed-phase HPLC fraction, several peptides indicated by arrows were collected and subjected to sequencing analysis.

TABLE I. **Sequences of Dm-calpain substrates and their identification.**

Band	Fraction No.	Sequence determined	Assignment; residue position	
A	8	FEAILE···	Partial sequence of α subunit of elongation factor-1	; 372nd-
	15	SVEMHHEALQLAX···	//	; 291st-
	24	NVSVKELRRGYVAGD···	//	; 314th-
C	15	EFNAEVHR···	Partial sequence of 60S ribosomal protein L5	; 188th-
	17	GAVDGGLSI PHST···	//	; 164th-
D	15	NFGI GQNVQP···	Partial sequence of 60S ribosomal protein L7A	; 43rd-
	17	VPPPI HQFSQ···	//	; 81st-
E	8	PGDRG···	Partial sequence of 60S ribosomal protein L8	; 119th-
	10	RGVAMNPVE···	//	; 199th-
	15	DI I HDPGRGAP···	//	; 47th-

The partial sequences of isolated peptides shown in Fig. 3 are shown together with their identification as revealed by database search.

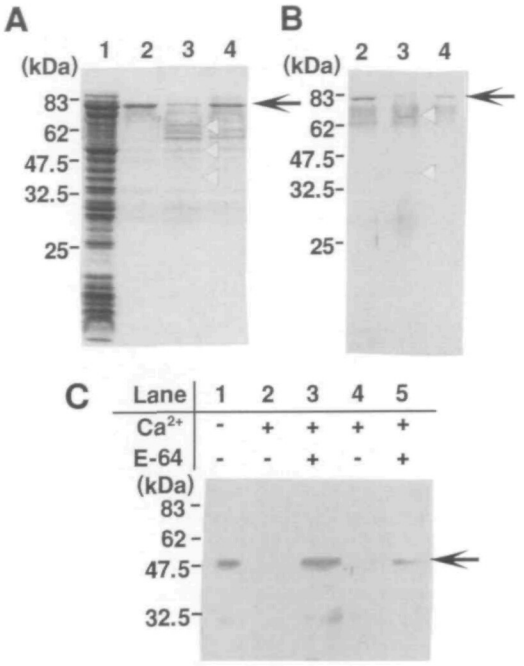

Fig. 5. **Degradation of EF-1α by recombinant Dm-calpain.** Degradation of GST-fusion EF-1α (A and B) and endogenous EF-1α in ovary (C) by Dm-calpain is shown. A: Coomassie Brilliant Blue staining; and B: immunoblot analysis using anti-EF-1α [Miyazaki *et al.*, 1988 (*40*)]. Lane 1, crude extract of *E. coli* producing GST-fusion EF-1α; lane 2, GST-EF-1α affinity-purified on glutathione Sepharose 4B; lane 3, GST-EF-1α incubated with Dm-calpain in the presence of 10 mM calcium ion; lane 4, GST-EF-1α incubated with Dm-calpain in the presence of 10 mM calcium ion and 0.5 mM E64. Degradation fragments of GST-EF-1α were observed in lane 3 of panel A (indicated by open triangles), and two of them were also recognized by anti EF-1α (indicated by an open triangle). C: Degradation of endogenous EF-1α in *Drosophila* ovary extracts prepared as described in "EXPERIMENTAL PROCEDURES." The soluble (lanes 1, 2, and 3) and salt-extracted (lanes 4 and 5) fractions were incubated with recombinant Dm-calpain in the presence (lanes 3 and 5) and absence (lanes 2 and 4) of E-64.

four panels of Fig. 4) were subjected to amino acid sequencing. As shown in Table I, the amino acid sequences of three peptides from bands A and E, and two peptides from bands C and D were determined. However, the sequences of the peptides from bands B and F could not be determined definitely. The sequences of peptides from the four bands (A, C, D, E) out of six major substrates (A-F) were subjected to a computer search using a protein sequence data base (Swiss-prot., Ref. *34*). As a result, the sequences of all ten peptides from the four proteins were clearly assigned to known sequences (Table I). Consequently, bands A, C, D, and E were shown to be the α subunit of elongation factor-1 (EF-1α), and ribosomal proteins L5, L7, and L8 of the 60S subunit, respectively (*26, 34-36*).

Limited Proteolysis of EF-1α by Dm-Calpain—EF-1α is known to interact with both actin and microtubule structures, to have bundling or severing activity against them, and to play a role as a translational component (*37-39*). Thus, we examined the mode of Dm-calpain action on *Drosophila* EF-1α using a recombinant EF-1α synthesized as a GST-fusion protein. As shown in Fig. 5A, GST-fusion *Drosophila* EF-1α affinity-purified on glutathione Sepharose 4B, was degraded by Dm-calpain into several fragments in a Ca^{2+}-dependent manner (lane 3, indicated by open triangles), but not degraded when a cysteine protease inhibitor, E64, was added to the reaction mixture (lane 4). To identify the degraded fragments of EF-1α, polyclonal antiserum against yeast EF-1α (*40*) was used to detect *Drosophila* EF-1α, since yeast EF-1α and *Drosophila* EF-1α show high sequence similarity (86%) and the antiserum cross-reacts with *Drosophila* EF-1α. Western blot analysis showed GST-fusion *Drosophila* EF-1α to be detected by the serum at the expected position (78 kDa; indicated by an arrow), and the degraded fragments of GST-fusion EF-1α were also recognized (Fig. 5B, lane 3; indicated by open triangles), showing that these fragments are not derived from *E. coli* proteins or autolysed calpain but from EF-1α. Furthermore, endogenous EF-1α in ovary extracts was also degraded by Dm-calpain (Fig. 5C, lanes 2 and 4), although we could not detect the degradation products, probably due to the high background and the lower endogenous EF-1α content than in the *E. coli* expression system.

DISCUSSION

Expression of Active Dm-Calpain in E. coli—For structural and enzymatic studies, it is important to obtain large quantities of the proteins of interest in an active form as simply as possible. Bacterial expression, especially in *E. coli*, is one of the simplest ways to achieve this purpose. In this study, we succeeded in producing active Dm-calpain in *E. coli*. This is the first report describing the sole expression of the calpain catalytic subunit in *E. coli* in an active form (*41*). In the case of mammalian μ- and m-calpains, which consist of 80 kDa catalytic and 30 kDa regulatory subunits, the co-existence of both subunits seems to be essential for proteolytic activity (*42*), although the cata-

lytic subunit of mammalian calpains reconstituted in the presence of polyethylene glycol has been shown to have full proteolytic activity (*43*). However, since no small subunit has been detected in Dm-calpain preparations (*33* and Emori, Y., unpublished observations), and since our expression study here showed no requirement for Dm-calpain activity other than the catalytic subunit, it appears that no subunit corresponding to the small subunit of mammal calpains is present in Dm-calpain. Considering that the apparent M_r of purified *Drosophila* calpain as estimated by gel filtration is 280,000 (*44*), which is more than twice the calculated M_r of Dm-calpain, Dm-calpain probably exists as a dimer or trimer, as previously suggested (*44*), or interacts with other factors not essential for the proteolytic activity and proper folding of Dm-calpain.

Several Components of the Protein Synthesis Machinery Are Selectively Degraded by Dm-Calpain—Recently, several calpain-like molecules, whose catalytic domains show significant homologies to those of mammal calpains, have been identified in various invertebrates (*18-20, 22-25*). This suggests that the calpain system is probably conserved in mammals and invertebrates. However, biochemical information about invertebrate calpains, such as their potential substrates and catalytic modes, had not previously been elucidated, and their physiological functions *in vivo* remain to be analyzed, except for *tra3* from *C. elegans* (*21*). We previously identified Dm-calpain, whose amino acid sequence is highly homologous to those of mammalian typical calpains, and showed that it appears to be involved in early embryogenesis, especially in the organization of the actin-related cytoskeleton (*23*). Here, we identified several candidate Dm-calpain substrates among the major proteins present in *Drosophila* ovaries using the recombinant active enzyme. These candidates include the L5, L7, and L8 subunits of the 60S ribosome and the α subunit of elongation factor-1 (EF-1α). Since only abundant proteins present in large amounts can be identified as being susceptible to Ca^{2+}-dependent degradation by Dm-calpain, while minor substrates are not easily detected by the methods employed here, it is likely that Dm-calpain also degrades various proteins other than the components of the protein synthesis machinery that are usually abundant in these cells. In fact, we detected other minor proteins that are cleaved by Dm-calpain in a Ca^{2+}-dependent manner (Fig. 3), and we have preliminarily identified cytoskeletal proteins as Dm-calpain substrates (Amano, S. and Emori, Y., unpublished results). In this respect, the identification of EF-1α as a substrate is noteworthy, because EF-1α is known to interact with actin filaments and the microtubule network both *in vitro* and *in vivo* (*37-39*). This is in addition to its well-known function as an important component of the protein synthetic machinery catalyzing the GTP-dependent binding of aminoacyl-transfer RNA to ribosomes (*45*). The finding that Dm-calpain can hydrolyze EF-1α in a limited manner suggests that Dm-calpain is also involved in EF-1α-mediated cytoskeletal changes. In addition, it should be noted that rabbit *m*-calpain, like Dm-calpain, also degrades mammalian EF-1α (Amano, S. and Emori, Y., unpublished results). This suggests that the substrate specificity of Dm-calpain, possibly together with its physiological significance, is closely related to that of mammal calpains, although the meaning of the limited proteolysis of EF-1α *in vivo* is not clear. Taken together, our observations support the view that Dm-calpain acts as a regulator of protein synthesis and/or the cytoskeleton by processing or down-regulating their components.

As described above, we have identified several potential substrates of an invertebrate calpain, Dm-calpain, by establishing an expression system for Dm-calpain cDNA. This approach should be useful for studies on other calpains and calpain-related proteases from both vertebrates and invertebrates, which, up to now, have been identified only at the cDNA or gene level. Cumulative studies on many calpains and related proteins using methods similar to ours, together with genetic approaches that can be carried out in specific animals such as *D. melanogaster* and *C. elegans*, will reveal the physiological functions of the calpain family.

We thank Prof. Masazumi Miyazaki of Nagoya Univ. and Prof. Takashi Saito of Chiba Univ. for providing anti-yeast EF-1α antibody and *E. coli* strain AD202, respectively, and Kayoko Kinbara for valuable discussion.

REFERENCES

1. Suzuki, K., Ohno, S., Emori, Y., Imajoh, S., and Kawasaki, H. (1987) Primary structure and evolution of calcium-activated neutral protease (CANP). *J. Protein Chem.* **6**, 7-15
2. Sorimachi, H., Saido, C.T., and Suzuki, K. (1994) New era of calpain research. Discovery of tissue-specific calpain. *FEBS Lett.* **343**, 1-5
3. Watt, F. and Molloy, P.L. (1993) Specific cleavage of transcription factors by the thiol protease, m-calpain. *Nucleic Acids Res.* **21**, 5092-5100
4. Hirai, S., Kawasaki, H., Yaniv, M., and Suzuki, K. (1991) Degradation of transcription factor, c-Jun and c-Fos, by calpain. *FEBS Lett.* **287**, 57-61
5. Kosaki, G., Tsujinaka, T., Kambayashi, J., Morimoto, K., Yamamoto, K., Yamagami, K., Sobue, K., and Kakiuchi, S. (1983) Specific cleavage of calmodulin-binding proteins by low Ca^{2+}-requiring form of Ca^{2+}-activated neutral protease in human platelets. *Biochem. Int.* **6**, 767-775
6. Wang, K.K., Villalobo, A., and Roufogalis, B.D. (1989) Calmodulin-binding proteins as calpain substrates. *Biochem. J.* **262**, 693-706
7. Magnusson, A., Haug, L.S., Walaas, S.I., and Ostvold, A.C. (1993) Calcium-induced degradation of the inositol (1,4,5)-trisphosphate receptor/Ca^{2+}-channel. *FEBS Lett.* **323**, 229-232
8. Fischer, I., Romano-Clarke, G., and Grynspan, F. (1991) Calpain-mediated proteolysis of microtubule associated proteins MAP1B and MAP2 in developing brain. *Neurochem. Res.* **16**, 891-898
9. Selliah, N., Brooks, W.H., and Roszman, T.L. (1996) Proteolytic cleavage of alpha-actinin by calpain in T cells stimulated with anti-CD3 monoclonal antibody. *J. Immunol.* **156**, 3215-3221
10. Schoenwaelder, S.M., Yuan, Y., Cooray, P., Salem, H.H., and Jackson, S.P. (1997) Calpain cleavage of focal adhesion proteins regulates the cytoskeletal attachment of integrin alphaIIbbeta3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin clots. *J. Biol. Chem.* **272**, 1694-1702
11. Emori, Y., Kawasaki, H., Sugihara, H., Imajoh, S., Kawashima, S., and Suzuki, K. (1986) Isolation and sequence analyses of cDNA clones for the large subunits of two isozymes of rabbit calcium-dependent protease. *J. Biol. Chem.* **261**, 9465-9471
12. Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M., and Suzuki, K. (1984) Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? *Nature* **312**, 566-570
13. Aoki, K., Imajoh, S., Ohno, S., Emori, Y., Koike, M., Kosaki, G., and Suzuki, K. (1986) Complete amino acid sequence of the large subunit of the low-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease (muCANP) deduced from its cDNA sequence. *FEBS Lett.* **205**, 313-317
14. Imajoh, S., Aoki, K., Ohno, S., Emori, Y., Kawasaki, H.,

Sugihara, H., and Suzuki, K. (1988) Molecular cloning of the cDNA for the large subunit of the high-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease. *Biochemistry* **27**, 8122-8128
15. Sorimachi, H., Imajoh-ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and mu-types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* **264**, 20106-20111
16. Sorimachi, H., Tayama-Sorimachi, N., Saido, T.C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S., and Suzuki, K. (1993) Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. *J. Biol. Chem.* **268**, 10593-10605
17. Sorimachi, H., Ishiura, S., and Suzuki, K. (1993) A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. *J. Biol. Chem.* **268**, 19476-19482
18. Andersen, K., Tom, T.D., and Strand, M. (1991) Characterization of cDNA clones encoding a novel calcium-activated neutral proteinase from *Schistosoma mansoni*. *J. Biol. Chem.* **266**, 15085-15090
19. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fraser, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johnston, L., Jones, M., Kershaw, J., Kirsten, J., Laisster, N., Latreille, P., Lightning, J., Lloyd, C., Mortimore, B., O'Callaghan, M., Parsons, J., Percy, C., Rifken, L., Roopra, A., Saunders, D., Shownkeen, R., Sims, M., Smaldon, N., Smith, A., Smith, M., Sounhammer, E., Staden, R., Sulston, J., Thierry-Mieg, J., Thomas, K., Vaudin, M., Vaughan, K., Wasterston, R., Watson, A., Weinstock, L., Wilkinson-Sproad, J., and Wohldman, P. (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature* **368**, 32-38
20. Wasterston, R., Martini, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shownkeen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berk, M., Du, Z., Thomas, K., Thierry-Meig, J., and Sulston, J. (1992) The *C. elegans* genome sequencing project. *Nature Genet.* **1**, 114-123
21. Barnes, M.T. and Hodgkin, J. (1996) The tra-3 sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. *EMBO J.* **15**, 4477-4484
22. Delaney, S.J., Hayward, D.C., Barleben, F., Fichbach, K.F., and Miklos, G.L.G (1991) Molecular cloning and analysis of small optic lobes, a structural brain gene of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **88**, 7214-7218
23. Emori, Y. and Saigo, K. (1994) Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. *J. Biol. Chem.* **269**, 25137-25142
24. Theopold, U., Pinter, M., Daffre, S., Tyselius, Y., Friedrich, P., Nassel, D.R., and Hultmark, D. (1995) CalpA, a *Drosophila* calpain homolog specifically expressed in a small set of nerve, midgut, and blood cells. *Mol. Cell. Biol.* **15**, 824-834
25. Denison, S.H. (1995) Signaling of ambient pH in *Aspergillus* involves a cysteine protease. *J. Biol. Chem.* **270**, 28519-28522
26. Hovemann, B., Richter, S., Walldorf, U., and Cziepluch, C. (1988) Two genes encode related cytoplasmic elongation factors 1 alpha (EF-1 alpha) in *Drosophila melanogaster* with continuous and stage. *Nucleic Acids Res.* **16**, 3175-3194
27. Nakano, H., Yamazaki, T., Ikeda, M., Masai, H., Miyatake, S., and Saito, T. (1994) Purification of glutathione S-transferase fusion proteins as a non-degraded form by using a protease-negative *E. coli* strain, AD202. *Nucleic Acids Res.* **22**, 543-544
28. Fyrberg, A.E. and Goldstein, S.B.L (1994) *Methods in Cell Biology*, Vol. 44, Academic Press, San Diego
29. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
30. Kawasaki, H., Emori, Y., and Suzuki, K. (1990) Production and separation of peptides from proteins stained with Coomassie brilliant blue R-250 after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **191**, 332-336
31. Towbin, H., Staehlin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4355
32. Matsumoto, I., Emori, Y., Abe, K., and Arai, S. (1997) Characterization of a gene family encoding cysteine proteinases of *Sitophilus zeamais* (maize weevil), and analysis of the protein distribution in various tissues including alimentary tract and germ cells. *J. Biochem.* **121**, 464-476
33. Pintér, M., Stierandova, A., and Friedrich, P. (1992) Purification and characterization of a Ca^{2+}-activated thiol protease from *Drosophila melanogaster*. *Biochemistry* **31**, 8201-8206
34. Frigerio, J.M., Dagorn, J.C., and Iovanna, J.M. (1995) Cloning, sequencing and expression of the L5, L21, L27a, L28, S5, S9, S10 and S29 human ribosomal protein mRNAs. *Biochim. Biophys. Acta* **1262**, 64-68
35. Armes, N. and Fried, M. (1995) The genomic organization of the region containing the *Drosophila melanogaster* rpL7a (Surf-3) gene differs from those of the mammalian and avian Surfeit loci. *Mol. Cell. Biol.* **15**, 2367-2373
36. Hanes, J., Klaudiny, J., Von Der Kammer, H., and Scheit, K.H. (1993) Characterization by cDNA cloning of the mRNA of human ribosomal protein L8. *Biochem. Biophys. Res. Commun.* **197**, 1223-1228
37. Yang, F., Demma, M., Warren, V., Daharmawardhane, S., and Condeelis, J. (1990) Identification of an actin-binding protein from Dictyostelium as elongation factor 1a. *Nature* **347**, 494-496
38. Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A., and Nishida, E. (1994) Microtubule severing by elongation factor 1 alpha. *Science* **266**, 282-285
39. Condeelis, J. (1995) Elongation factor 1 alpha, translation and the cytoskeleton. *Trends Biochem. Sci.* **233**, 169-215
40. Miyazaki, M., Uritani, M., Fujimura, K., Yamakatsu, H., Kageyama, T., and Takahashi, K. (1988) Peptide elongation factor 1 from yeasts: purification and biochemical characterization of peptide elongation factors 1 alpha and 1 beta (gamma) from *Saccharomyces carlsbergensis* and *Schizosaccharomyces pombe*. *J. Biochem.* **103**, 508-521
41. Elce, J.S., Hegadorn, C., Gauthier, S., Vince, J.W., and Davies, P.L. (1995) Recombinant calpain II: improved expression systems and production of a C105A active-site mutant for crystallography. *Protein Eng.* **8**, 843-848
42. Graham, S.K., Gauthier, S., Davies, L.P., and Elce, S.J. (1994) Active recombinant rat calpain II. Bacterially produced large and small subunits associate both in vivo and in vitro. *J. Biol. Chem.* **269**, 30457-30460
43. Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) A catalytic subunit of calpain possesses full proteolytic activity. *FEBS Lett.* **358**, 101-103
44. Pintér, M. and Friedrich, P. (1988) The calcium-dependent proteolytic system calpain-calpastatin in *Drosophila melanogaster*. *Biochem. J.* **253**, 467-473
45. Slobin, L. (1980) The role of eucaryotic factor Tu in protein synthesis. The measurement of the elongation factor Tu content of rabbit reticulocytes and other mammalian cells by a sensitive radioimmunoassay. *Eur. J. Biochem.* **110**, 555-563