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cDNA Cloning of Mouse Prolyl Endopeptidase and Its Involvement in DNA Synthesis by Swiss 3T3 Cells[1]

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A cDNA for mouse prolyl endopeptidase (PEP) was cloned and its nucleotide sequence determined. The overall amino acid sequence identity between mouse and other mammalian PEPs was about 96%. A specific inhibitor of PEP, *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA), inhibited DNA synthesis by Swiss 3T3 cells. Mouse PEP was shown to be localized partly in restricted nuclear regions. These results suggest that PEP participates in mammalian DNA synthesis.

Key words: DNA synthesis, nuclear localization, prolyl endopeptidase, Swiss 3T3 cells.

Prolyl endopeptidase (PEP), a proteinase that cleaves the carboxyl side of proline residues in peptides, is found in various organisms, including animals (*1-11*), plants (*12*), mushrooms (*13, 14*), and bacteria (*15-18*). PEP is a serine proteinase and it contains a consensus amino acid sequence, D-x(3)-A-x(3)-[LIVMFYW]-x(14)-G-x-S-x-G-G-[LIVMFYW], but its overall amino acid sequence clearly differs from those of typical serine proteinases such as trypsin and subtilisin (*19, 20*). Although PEP is likely to be ubiquitous in tissues, its biological role is totally unknown.

In previous studies, we demonstrated that various serine and cysteine proteinase inhibitors interfere with the differentiation of imaginal discs of *Sarcophaga peregrina* (flesh fly), when their differentiation was examined *in vitro* by culturing them in the presence of 20-hydroxyecdysone (20-HE) (*21*). We identified cathepsin L as a cysteine proteinase participating in imaginal disc differentiation, and demonstrated that it is secreted from the imaginal discs and digests specific substrate proteins in the disc basement membrane during disc differentiation (*22*). We also found that PEP is essential for imaginal disc differentiation (*21*).

We purified a PEP from an embryonic cell line of *Sarcophaga* and determined its complete amino acid sequence by isolating its cDNA (*22, 23*). A specific inhibitor of PEP, *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA) (*24, 25*), was found to inhibit DNA synthesis in the embryonic cells and imaginal discs cultured in the presence of 20-HE (*22, 26*). Moreover, *Sarcophaga* PEP was localized exclusively in the nuclei in the growing embryonic cells (*26*). These results suggest that PEP participates in DNA synthesis by, and therefore cell proliferation of, insect cells.

To extend these findings to other organisms, we have conducted similar experiments with mouse Swiss 3T3 cells. We report here that ZTTA inhibited the proliferation of Swiss 3T3 cells. Furthermore, mouse PEP was localized partly in the nuclei. These results are essentially the same as those obtained with insect cells, suggesting that PEP participates in the essential process of DNA replication.

MATERIALS AND METHODS

Cells—Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cloning and Sequencing of Mouse PEP cDNA—A probe for screening a mouse brain cDNA library was obtained by reverse transcription-polymerase chain reaction (RT-PCR) and by the 5′-rapid amplification of cDNA-end method, from the total RNA extracted from a mouse brain. DNA primers were designed from amino acid sequences conserved between human and pig PEPs. These were MLSFQYPD and WYCDLQQE for the initial PCR and RGLYKERMT and MAWTHDGK for nested PCR. The PCR product was cloned and sequenced. From this sequence, primers for use with a commercial cDNA amplification kit (Marathon cDNA amplification Kit; Clontech) were designed. Their sequences were 5′-CCGGGAGCTCTTTGGCACCATCGACC-3′ (primer 1 for the initial PCR with adapter primer 1) and 5′-GGCCTCCCCCTCTAAGGAGTCTTGCAC-3′ (primer 2 for the nested PCR with adapter primer 2). The resulting PCR product was cloned and used as a probe for cloning PEP cDNA. The probe was labeled with [α-^{32}P]-dCTP using a random primer labeling kit (the BcaBEST; Takara, Kyoto). One positive clone was isolated from 1×

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Abbreviations: 20-HE, 20-hydroxyecdysone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MCA, methylcoumaryl-7-amide; PCR, polymerase chain reaction; PEP, prolyl endopeptidase; PI, propidine iodide; SDS, sodium dodecyl sulfate; Suc, succinyl; ZTTA, *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal.

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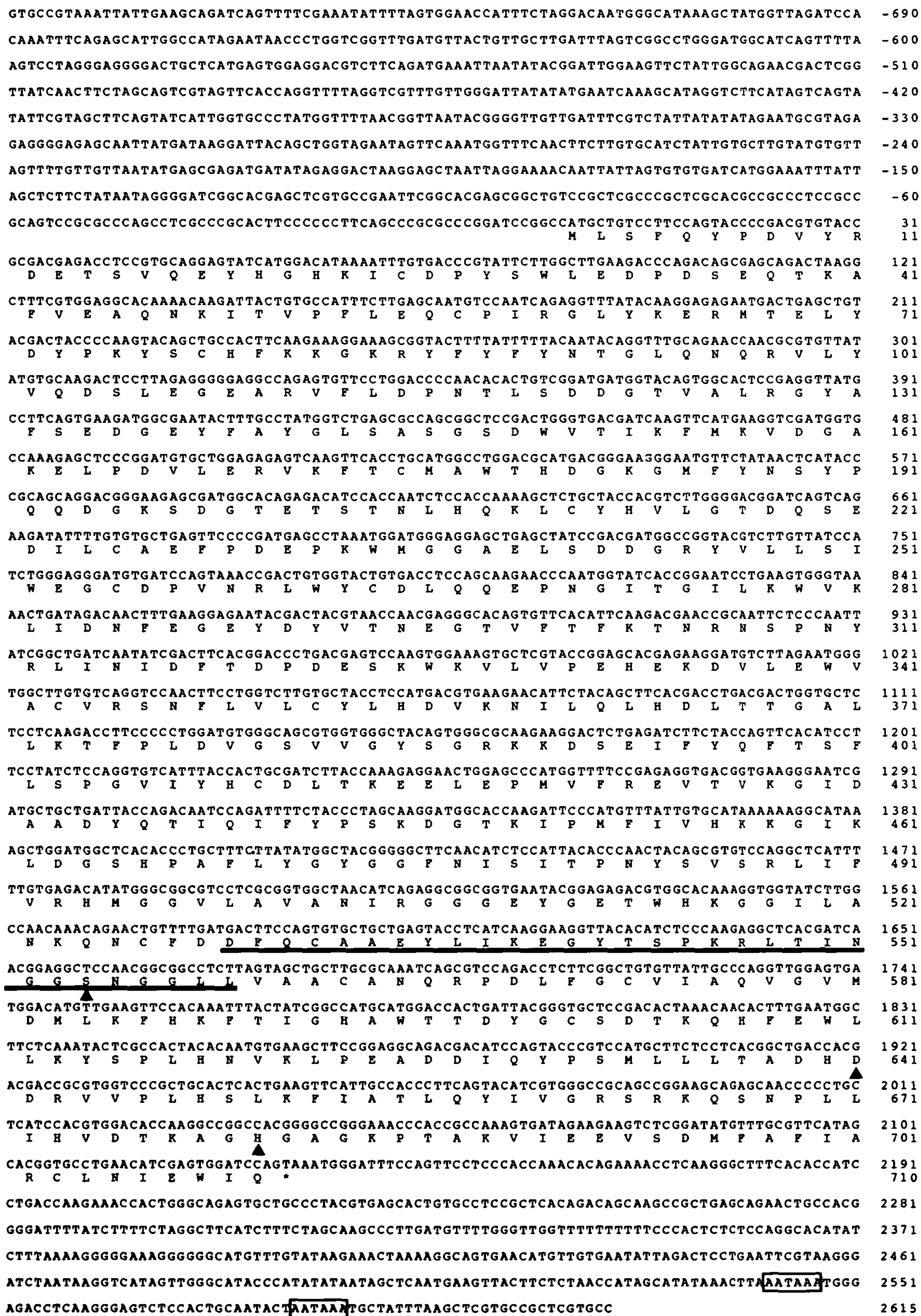

Fig. 1. **Nucleotide and deduced amino acid sequences of mouse PEP.** The deduced amino acid sequences is shown below the nucleotide sequence. The amino acid residues are numbered starting from the first Met residue, the nucleotide numbers start from the first letter of the Met codon. The asterisk shows the termination codon. The prolyl endopeptidase family serine active site is underlined, and the Ser, Asp, and His residues essential for enzyme activity are indicated by triangles. Polyadenylation signals are boxed.

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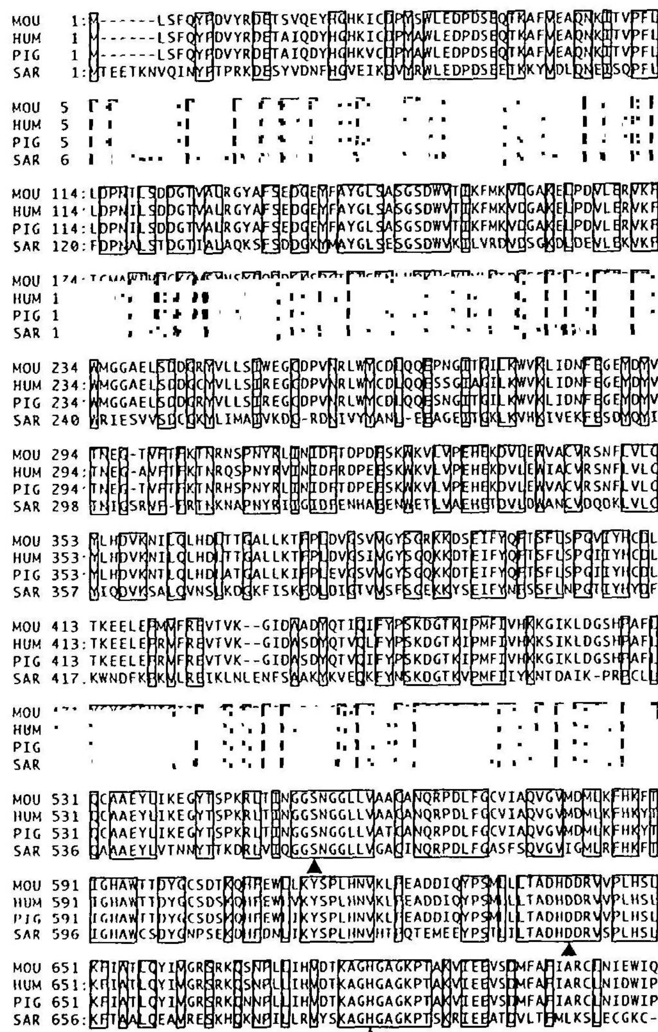

Fig. 2. **Comparison of the amino acid sequences of mouse and other PEPs.** The amino acid sequence of mouse PEP (MOU) is compared with those of human (HUM), pig (PIG), and *Sarcophaga* (SAR) PEPs. Gaps were inserted for optimal matching. The amino acid numbers from the first Met residue are shown on the left of each line, residues conserved in all four sequences are boxed and the residues essential for enzyme activity are indicated by triangles.

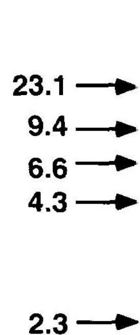

Fig. 3. **Southern blot hybridization of mouse genomic DNA.** Mouse genomic DNA (4 μg) was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Bam*HI (lane 3), *Pst*I (lane 4), and *Bgl*II (lane 5). Numbers on the left indicate mobilities of *Hin*dIII-digested lambda DNA fragments in kilobases.

10^5 clones of a mouse brain cDNA library (Uni-ZAP; C57 Black/6, female, 19 weeks; Stratagene) by colony hybridization. It was sequenced by the dideoxy chain-termination method (*27*) using an automatic DNA sequencer (Applied Biosystems), and the nucleotide sequences of both strands were determined.

Southern Blot Hybridization—Southern blot hybridization was performed in 50% formamide, 5×SSPE (0.75 M NaCl, 43.25 mM NaH_2PO_4-$2H_2O$, 25 mM EDTA), 10× Denhardt's solution [0.2% (w/v) each of Ficoll-400, polyvinyl pyrrolidone, and bovine serum albumin], 2% sodium

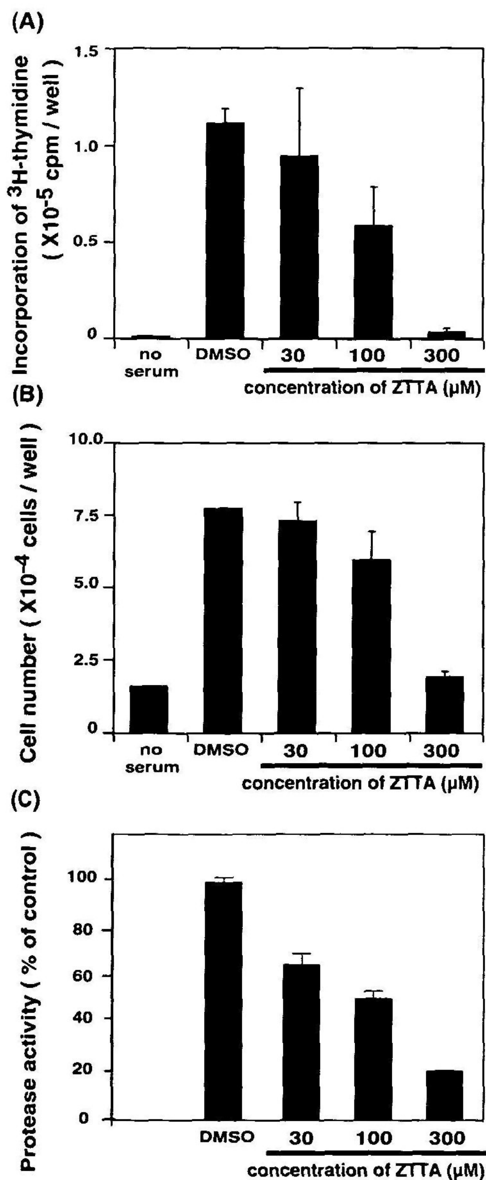

Fig. 4. **Effect of ZTTA on Swiss 3T3 cells.** Swiss 3T3 cells were inoculated into 5% FCS in DMEM and cultured at 37°C in the presence of 30, 100, and 300 μM ZTTA, respectively. As a negative control, DMSO alone was added to the medium. (A) After incubation for 24 h, [^{3}H]thymidine was added and the cells were labeled for 24 h. The radioactivity incorporated into the acid-insoluble fraction was measured. (B) After incubation for 48 h, the cells were counted. (C) After incubation for 24 h, cells were harvested, and PEP activity in the cell extracts was measured.

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dodecyl sulfate (SDS), sonicated salmon sperm DNA solution (100 μg/ml) for 18 h at 65°C. The probe used was the same as for PEP cDNA cloning. Then the filter (Mouse Geno-Blot; Clontech) was washed with 2×SSC (33.3 mM NaCl, 33.3 mM $C_6H_5O_7Na_3$-$2H_2O$) containing 0.05% SDS successively at room temperature and at 60°C. Then the filter was subjected to autoradiography at −80°C.

Measurements of DNA Synthesis and PEP Activity—Swiss 3T3 cells were inoculated in 5% FCS in DMEM at a density of 1.5×10^4 cells/well and cultured overnight at 37°C in 5% CO_2. Then ZTTA dissolved in dimethylsulfoxide (DMSO) or DMSO alone was added to the medium. After incubation for another 24 h, 16 μl of medium containing 1.6 μCi of [^{3}H]thymidine was added to each well and the incubation was continued for 24 h to label the DNA. Then the cells were washed well, lysed in 0.5 N NaOH solution, and trichloroacetic acid was added to the lysate at a final concentration of 30% (v/v). The acid-insoluble material was trapped on a glass fiber filter (Whatman, GF/C) and its radioactivity was measured to quantify DNA synthesis. To assay PEP activity in the cell extracts, cells treated with ZTTA for 24 h were harvested, washed well, homogenized in 50 mM phosphate buffer (pH 7.5) containing 100 mM NaCl, and then centrifuged at 100,000×g for 30 min. PEP activity in the resulting supernatants was assayed essentially as described previously using succinyl (Suc)-Gly-Pro-methylcoumaryl-7-amide (MCA) substrate (*21*). After incubation for 10 min at 37 °C, the reactions were terminated by adding 0.2 ml of 20% aqueous acetic acid and fluorescence intensity was measured at excitation and emission wavelengths of 380 and 460 nm, respectively.

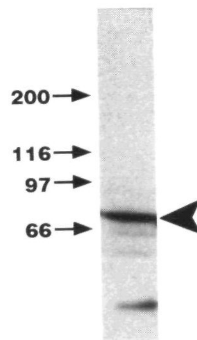

Fig. 5. **Detection of PEP in Swiss 3T3 cell lysate.** The lysate of Swiss 3T3 cells (40 μg protein) was subjected to SDS polyacrylamide gel electrophoresis and then immunoblotting with an antiserum against a 30-residue peptide derived from mouse PEP. The arrowhead indicates a 69-kDa protein assumed to be mouse PEP. Numbers on the left indicate the mobilities of molecular-mass markers in kilodaltons: a, myosin heavy chain (200 kDa); b, β-galactosidase (116 kDa); c, phosophorylase *b* (97 kDa); d, bovine serum albumin (66 kDa).

Preparation of Antiserum against Mouse PEP and Immunoblotting—Antibody was raised against a peptide derived from mouse PEP essentially as described before. A 30-residue peptide, located from position 190 (Tyr) to 219 (Gln) of mouse PEP, was synthesized by PSSM-8 (Shimadzu), conjugated to keyhole limpet hemocyanin, and injected into a female albino rabbit. Immunoblotting was performed essentially as described previously (*28*). Briefly, proteins separated by polyacrylamide gel electrophoresis were transfered onto polyvinylidene difluoride membrane filters, and the filters were treated with antiserum against the PEP peptide followed by treatment with peroxidase-linked anti-rabbit donky IgG. The filters were then treated with ECL Western blotting detection reagents (Amersham Life Science) and exposed to film.

Immunofluorescence Study—Swiss 3T3 cells (5×10^4 cells/ml) were seeded onto a glass slides and cultured for 2 days to allow the cells adhere to the glass. The medium was discarded, and the adherent cells were washed with saline and fixed with 5% acetic acid/90% ethanol for 30 min. Then, the cells were blocked with 1% FCS and treated successively with an antiserum against the PEP peptide and

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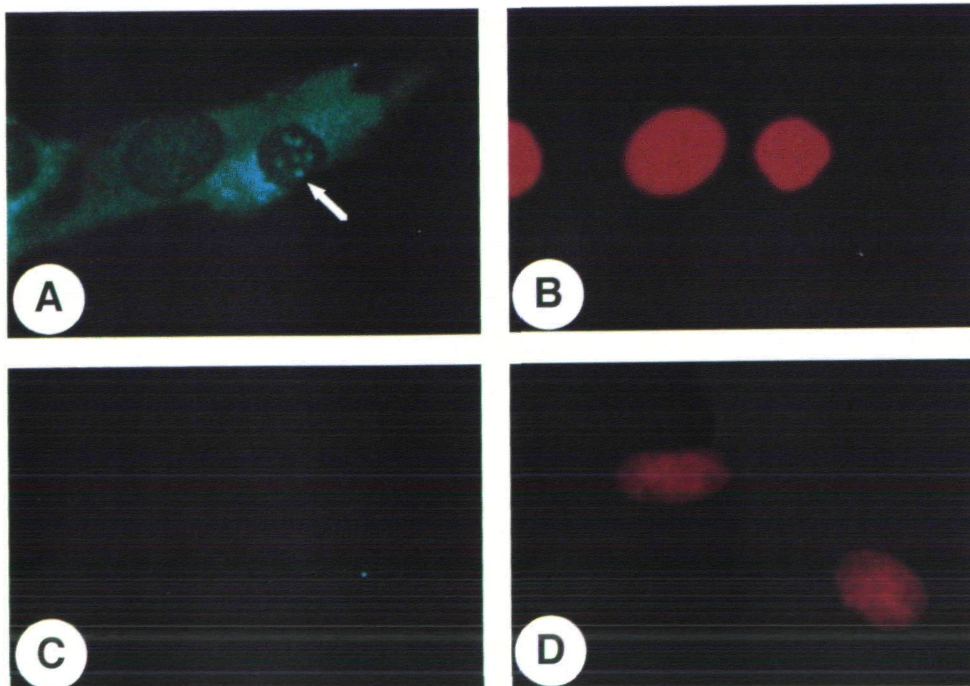

Fig. 6. **Immunofluorescence study of PEP.** Swiss 3T3 cells were cultured on glass slides, fixed with 5% acetic acid/90% ethanol and treated antibody against a PEP-derived peptide followed by FITC-conjugated secondary antibody in a solution containing PI to locate the nuclei. Control cells were treated with a preimmune serum. (A) PEP immunofluorescence; (B) PI staining of (A); (C) sample treated with preimmune serum; (D) PI staining of (C). The arrow indicates heterogeneous localization of PEP in the nuclei.

fluorescein isothiocyanate (FITC)-conjugated anti-rabbit swine IgG solution, containing propidine iodide (PI) (500 ng/ml) to stain the DNA. Finally, the cells were immersed in 50% fluorescence-free glycerol containing 2.5% 1,4-diazabicyclo-[2,2,2]-octane and mounted for examination.

RESULTS

Molecular Cloning of cDNA for Mouse PEP and Southern Blotting Analysis—To study the biological roles of mammalian PEP in cell proliferation, we examined Swiss 3T3 cells. As mouse PEP has not been characterized, we first tried to isolate mouse PEP cDNA. To do this, we performed PCR to obtain a fragment of mouse PEP cDNA using nucleotide primers based on sequences that are highly conserved between human and pig PEP cDNA, and we finally isolated one hybridization-positive clone by screening about 1×10^5 colonies of a mouse brain cDNA library with this cDNA fragment as the probe. This clone contained an insert of about 3.5 kb that carries an open reading frame of 2,130 nucleotides corresponding to 710 amino acid residues. The nucleotide sequence together with the putative amino acid sequence encoded by this cDNA are shown in Fig. 1. This amino acid sequence was very similar to those of human, pig, and *Sarcophaga* PEPs as shown in Fig. 2. The amino acid identity between this protein and human and pig PEPs was 96%. Furthermore, three amino acid residues, Ser (544), Asp (641), and His (680), which are essential for serine proteinase activity were conserved in this sequence, indicating that this cDNA was for mouse PEP. To examine the organization of the mouse PEP gene, we performed Southern blot hybridization. A single hybridization-positive band was detected when mouse genomic DNA was digested with five independent restriction enzymes (Fig. 3). As the cDNA does not contain sites for these restriction enzymes, the mouse PEP gene is likely to be a single copy gene.

Participation of PEP in Proliferation of Swiss 3T3 Cells—Our previous study suggested that *Sarcophaga* PEP is involved in DNA synthesis when embryonic cells proliferate (*26*). To determine whether mouse PEP also participates in DNA synthesis, we examined the effect of ZTTA, a specific inhibitor of PEP, on DNA synthesis of Swiss 3T3 cells. DNA synthesis and expected increase in cell number of Swiss 3T3 cells were inhibited significantly when 30-300 μM ZTTA was added to the culture medium (Fig. 4, A and B), whereas DMSO alone had virtually no effect. The PEP activity in the extracts of ZTTA-treated cells was found to be inhibited depending on the concentration of ZTTA in the culture medium (Fig. 4C). These results were almost the same as those obtained with *Sarcophaga* embryonic cells. Similar inhibitory effects of ZTTA were detected with NIH 3T3 cells. Thus, in mammalian cells PEP is likely to be involved in DNA synthesis and cell proliferation as it is in insect cells.

Immunoblot Analysis and Immunofluorescence Study of Mouse PEP—To investigate the intracellular distribution of mouse PEP, we raised an antibody against a 30-residue peptide with a sequence that is well conserved among mammalian PEPs. When the lysate of Swiss 3T3 cells was subjected to immunoblot analysis using this antibody, a protein with molecular mass of 69 kDa was clearly detected, as shown in Fig. 5. The molecular mass of mouse PEP calculated on the basis of its cDNA sequence is about 80 kDa. Therefore, there is a discrepancy between these two molecular masses. Nonetheless, we concluded that the 69 kDa protein detected with this antibody is mouse PEP, since similar discrepancies between calculated and observed molecular masses have been reported for other mammalian PEPs (*8, 9*).

Using this antibody, we examined the distribution of mouse PEP in Swiss 3T3 cells by indirect immunofluorescent staining. As shown in Fig. 6A, fluorescence derived from mouse PEP was detected in both the cytoplasm and nuclei. No fluorescence was detected when preimmune serum was used (Fig. 6C). Although distribution of fluorescence in the cytoplasm was diffuse, that in the nuclei was heterogeneous. As indicated by an arrow, clear spots of fluorescence were detected in some nuclei, which were very similar to the PEP distribution in the nuclei of insect cells. These results indicate that PEP is present in both the cytoplasm and nuclei. PEP in the nuclei may be involved in a process of DNA synthesis that is common between insect and mammalian cells.

DISCUSSION

It has been suggested that PEP in mammals participates in the metabolism of proline-containing neuropeptides and hormones, such as substance P, bradykinin, angiotensin II, and thyrotropin-releasing hormone, because PEP digests these peptides *in vitro* (*29, 30*). PEP is also implicated in Alzheimer's disease (*31*) and in systemic lupus erythematosus (*32*), in humans. However, the biological role of PEP remains unknown. Previously, we showed that ZTTA selectively inhibits DNA synthesis in *Sarcophaga* cells and suggested that PEP is involved in DNA synthesis, and thus cell proliferation (*22, 26*). In fact, *Sarcophaga* PEP was detected exclusively in the nuclei when the cells were examined by indirect immunofluorescence. To extend these findings, we examined the effect of ZTTA on mouse cells and confirmed the results obtained with *Sarcophaga* cells. ZTTA was shown to inhibit PEP specifically when its effects on various proteinases were tested *in vitro* (*24*). Moreover, it is the only one of the PEP inhibitors that we have tested so far to be incorporated into cells. Our results suggest that PEP plays a crucial role in DNA synthesis, even in mammalian cells.

In *Sarcophaga* cells, PEP immunofluorescence was detected exclusively in the nuclei (*26*), whereas in mouse cells it was present in both the cytoplasm and nuclei. As mouse PEP is likely to be a single gene product, the same enzyme is playing different roles in different cellular compartments. To raise PEP antibody, we used a peptide derived from it. On immunoblotting with this antibody using crude cell lysate, we detected a major 69-kDa protein, that we assumed to be mouse PEP, and a few smaller proteins. These smaller proteins are likely to be degradation products of PEP. However, there remains a possibility that they are other proteins with the same peptide sequence as the one we used for raising the antibody. The distribution of PEP immunofluorescence in the nuclei was heterogeneous and it was very similar to the distribution of *Sarcophaga* PEP immunofluorescence (*26*).

Recently, it has been suggested that some proteinases are necessary for progression of the cell cycle, perhaps through

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the degradation of protein kinases or their inhibitors (*33*). Further molecular analysis is needed, including the identification of the nuclear substrate of PEP, to clarify the function of PEP in DNA synthesis.

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