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Nuclear Localization and Involvement in DNA Synthesis of *Sarcophaga* Prolyl Endopeptidase[1]

Sumio Ohtsuki, Ko-ichi Homma, Shoichiro Kurata, and Shunji Natori[2]

Faculty of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113

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A specific prolyl endopeptidase (PEP) inhibitor, ZTTA, selectively inhibited DNA synthesis by imaginal discs and cultured embryonic cells of *Sarcophaga peregrina* (flesh fly). PEP was found to be localized in restricted nuclear regions. Unfertilized eggs were shown to contain a maternal message for PEP and analysis of *Sarcophaga* embryos at blastdermal stage revealed PEP was localized exclusively in the nuclei. These results suggest that PEP participates in DNA synthesis by, and therefore cell proliferation, of insect cells. This is the first demonstration of a biological function of PEP.

Key words: DNA synthesis, imaginal disc, nuclear localization, prolyl endopeptidase, *Sarcophaga peregrina*.

Prolyl endopeptidase (PEP) is a ubiquitous serine protease found in various organisms, including animals (*1-9*), plants (*10*), mushrooms (*11, 12*), and bacteria (*13*). PEP is known to cleave certain oligopeptides at the carboxy side of their proline residues, but its biological role is not well known. In mammals, PEP has been suggested to participate in the metabolism of proline-containing neuropeptides and hormones, such as substance P, bradykinin, angiotensin II, and thyrotropin-releasing hormone, because it was found to digest these peptides *in vitro* (*14, 15*).

In a previous study, we purified a PEP from cultured embryonic cells of *Sarcophaga peregrina* (flesh fly) and determined its complete amino acid sequence by isolating its cDNA (*16*). Our experiments using a specific inhibitor of PEP, *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA) (*17, 18*), suggested that PEP participates in imaginal disc differentiation (*19*). When imaginal leg discs were cultured in the presence of 20-hydroxyecdysone (20-HE), they were shown to differentiate into adult structures *in vitro*. However, when ZTTA was present in the culture medium, their differentiation stopped at the stage of eversion, the initial stage of imaginal disc differentiation.

We have conducted further studies into the role of PEP in the development of *Sarcophaga*, and found that it is not only important for imaginal discs differentiation, but also plays a crucial role in the embryonic development of this insect. In this study, we demonstrated that ZTTA selectively inhibited proliferation of imaginal disc cells and embryonic cells, monitored by evaluating DNA synthesis. Moreover, *Sarcophaga* PEP was found to be localized exclusively in the nuclei. These results suggest strongly that PEP participates in the essential process of cell proliferation that takes place in the nuclei.

MATERIALS AND METHODS

Cells, Imaginal Discs, and Culture Media—The embryonic cell line, NIH-Sape-4, established from *Sarcophaga* was cultured in M-M medium at 25°C as described previously (*20*). Imaginal leg discs were prepared from third instar larvae and cultured in Grace's insect medium, as described previously (*21*).

Northern Blot Analysis—Samples (20 μg) of RNA were subjected to 1.2% formaldehyde-agarose gel electrophoresis followed by transfer of the RNA to nitrocellulose filters. Rat ribosomal RNA was electrophoresed simultaneously as a reference marker. The amount of *Sarcophaga* mRNA was normalized by that of 18 S ribosomal RNA in each RNA fraction. The filter was baked for 5 h at 80°C and prehybridized with the hybridization solution described below. The filters were hybridized in a solution comprising 50% formamide, 5×SSC, 1×Denhardt's solution, 50 mM phosphate buffer (pH 6.5), 0.1% SDS, and 200 μg/ml sonicated salmon sperm DNA for 18 h at 42°C. Then the filter was washed twice with 2×SSC containing 0.1% SDS for 15 min at room temperature and autoradiographed at −80°C. The DNA used as probe was the 1,922 bp *Bam*HI-*Bst*1107I fragment of the coding region of *Sarcophaga* PEP cDNA (*16*). The probe was labeled with [α-^{32}P]dCTP using the BcaBEST random primer labeling kit (Takara).

Quantification of Cell Proliferation and DNA Synthesis—NIH-Sape-4 cells were inoculated in 360 μl mixture comprising of 300 μl fresh M-M medium and 60 μl conditioned medium at a density of 4×10^5 cells/ml to facilitate cell growth. This mixture was transferred to a well, incubated at 25°C for 3 days and cells were counted. The conditioned medium was prepared exactly as described previously (*22*).

DNA synthesis was quantified by measuring the amount of [^{3}H]thymidine incorporated into DNA. After incubation

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[2] To whom correspondence should be addressed. Tel: +81-3-5684-2973, Fax: +81-3-3813-5099

for 18 h, 10 μl medium containing 1 μCi [^{3}H]thymidine was added to the above mixture and incubation was continued for 24 h to label DNA. Then the medium was discarded, the cells were suspended in 0.5 ml 0.5 N NaOH solution, 0.5 ml ice-cold 60% trichloroacetic acid solution was added, and the acid-insoluble material was trapped on a glass fiber filter (Whatman, GF/C) and its radioactivity was measured.

In order to quantify cell proliferation using imaginal discs, 20 leg discs were cultured in 500 μl Grace's insect medium in the presence or absence of 1 μM of 20-HE for the required time at 25°C. Then, 2 μCi [^{3}H]thymidine was added, the discs were labeled for 4 h, followed by disruption of the labeled discs in 0.5 ml 0.5 N NaOH solution and measurement of the radioactivity incorporated into the DNA of the acid-insoluble material.

Quantification of Protein Synthesis—Protein synthesis was quantified by measuring the amount of [^{35}S]methionine incorporated into newly synthesized protein. NIH-Sape-4 cells were inoculated into 500 μl methionine-free Grace's insect medium supplemented with 5% fetal calf serum at a density of 4×10^5 cells/ml. After incubation for 18 h at 25°C, [^{35}S]methionine (2 μCi) was added to the medium, incubation was continued for 2 h, the cells were harvested, suspended in 5% trichloroacetic acid, and the radioactivity of the acid-insoluble material was measured. When required, ZTTA dissolved in dimethylsulfoxide or dimethylsulfoxide alone, to produce the desired concentration, was added to the culture medium.

Immunoblotting—Immunoblotting was performed essen-

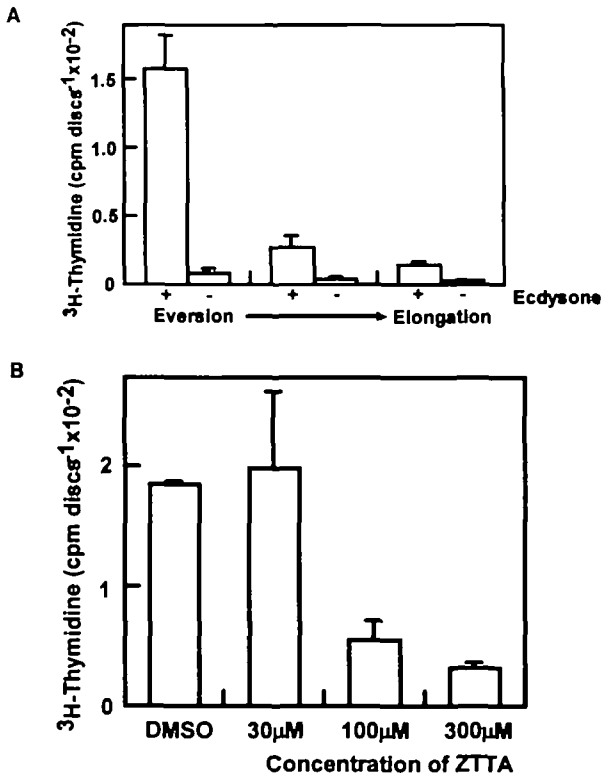

Fig. 1. **Inhibition by ZTTA of DNA synthesis of imaginal discs.** (A) Twenty leg discs were cultured in 500 μl Grace's insect medium in the presence or absence of 1 μM 20-HE at 25°C for 1 (column 1), 1.5 (column 2), and 2 (column 3) days. Then, 2 μCi [^{3}H]-thymidine was added, the discs were labeled for 4 h and DNA synthesis was evaluated. The developmental stages of discs cultured in the presence of 20-HE are shown at the bottom. (B) Imaginal discs were cultured in the presence of 20-HE with DMSO alone (column 1) or ZTTA (30, 100, 300 μM) dissolved in DMSO for 1 day, 2 μCi [^{3}H]-thymidine was added, the discs were labeled for 4 h and DNA synthesis was evaluated.

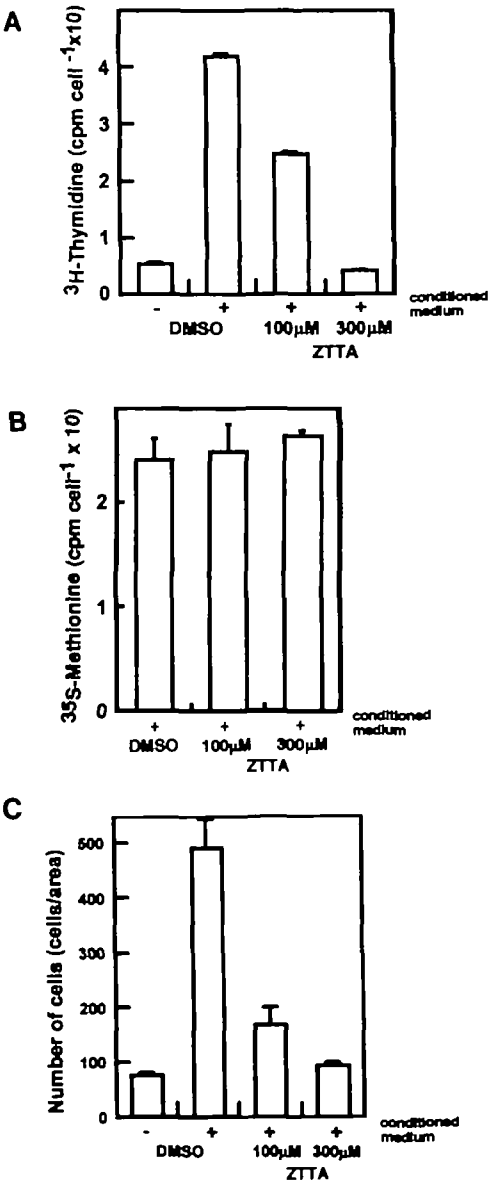

Fig. 2. **Effects of ZTTA on NIH-Sape-4 cells.** (A) NIH-Sape-4 cells were inoculated into a mixture of fresh M-M medium and conditioned medium and cultured at 25°C in the presence of 100 and 300 μM ZTTA. DMSO alone was added to the culture medium of control cells. The presence and absence of conditioned medium was shown with + and −, respectively. After incubation for 18 h, [^{3}H]-thymidine was added and the cells were labeled for 24 h, after which, the radioactivity incorporated into the acid-insoluble fraction was measured. (B) Cells were labeled with [^{35}S]methionine instead of [^{3}H]thymidine, for 2 h and the radioactivity of the acid-insoluble fraction was measured. (C) NIH-Sape-4 cells were inoculated into a mixture of fresh M-M medium and conditioned medium and cultured at 25°C in the presence of 100 and 300 μM ZTTA. DMSO alone was added to the culture medium of control cells. After incubation for 36 h, cells were counted.

tially as described previously (*23*). Briefly, proteins separated by polyacrylamide gel electrophoresis (*24*) were transferred electrophoretically from the gel onto polyvinylidene difluoride membrane filters, which were immersed in 5% skim milk solution for 1 h, transferred to rinse solution [10 mM Tris/HCl buffer (pH 7.9), containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100, and 0.25% skim milk] containing an affinity-purified antibody (IgG) against *Sarcophaga* PEP and kept at 4°C for 12 h. Then, they were washed well with the rinse solution, transferred to 5 ml rinse solution containing radioiodinated anti-rabbit sheep IgG (2×10^6 cpm) and kept for 2 h at room temperature. Finally they were washed thoroughly with rinse solution, dried and autoradiographed using Kodak XAR film.

Immunofluorescence Study—NIH-Sape-4 cells ($6\text{-}9\times10^5$ cells/ml) were seeded onto a coverslip, cultured for one day to make the cells adhere to it, after which, 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% fetal calf serum and treated successively with an affinity-purified antibody against *Sarcophaga* PEP (10 μg/ml IgG) for 1 h and FITC-conjugated anti-rabbit sheep Ig G solution containing DAPI (100 ng/ml). Propidine iodide (10 μg/ml) was added to this mixture, when needed, 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. In order to examine embryos, they were frozen in Tissue Tek, and 10-μm-thick sections were prepared on gelatin-coated, fluorescence-free glass slides, fixed with 1.5% formaldehyde for 10 min and then treated in the same way as the NIH-Sape-4 cells after fixation.

RESULTS

Participation of PEP in Cell Proliferation—Our previous study on *Sarcophaga* imaginal discs cultured in the presence of 20-HE *in vitro* indicated that PEP plays a crucial role in disc differentiation (*19*). Further study revealed that PEP is needed for cell proliferation. As shown in Fig. 1A, a significant DNA synthesis was detected only at the very early stage of eversion when leg discs were cultured *in vitro* in the presence of 20-HE, indicating that cell proliferation occurs only at this restricted stage. This DNA synthesis was found to be inhibited significantly when ZTTA was present in the culture medium (Fig. 1B), whereas dimethylsulfoxide (DMSO), the solvent of ZTTA, alone had virtually no effect. These results suggest that PEP participates in DNA replication. In order to confirm this, we examined the effect of ZTTA on DNA synthesis using another system, NIH-Sape-4 cells. As shown in Fig. 2, A and B, DNA, but not protein, synthesis by NIH-Sape-4 cells was inhibited specifically in the presence of ZTTA, the effect of which on DNA synthesis was essentially the same as that observed with imaginal discs, suggesting that PEP plays a role in DNA replication in insect cells. Cell proliferation assessed by counting cells was also shown to be inhibited under these conditions (Fig. 2C).

PEP Is Localized in Nuclei—As PEP appeared to participate in DNA synthesis, we examined its localization in growing NIH-Sape-4 cells using an indirect immunofluorescence method. As shown in Fig. 3, A and B, fluorescence derived from PEP was detected almost exclusively in nuclei that were visualized by DAPI staining. This fluorescence was not detected when the anti-PEP antibody was absorbed by recombinant PEP, as shown in Fig. 3, C and D. These results, we believe, are the first to indicate that PEP is

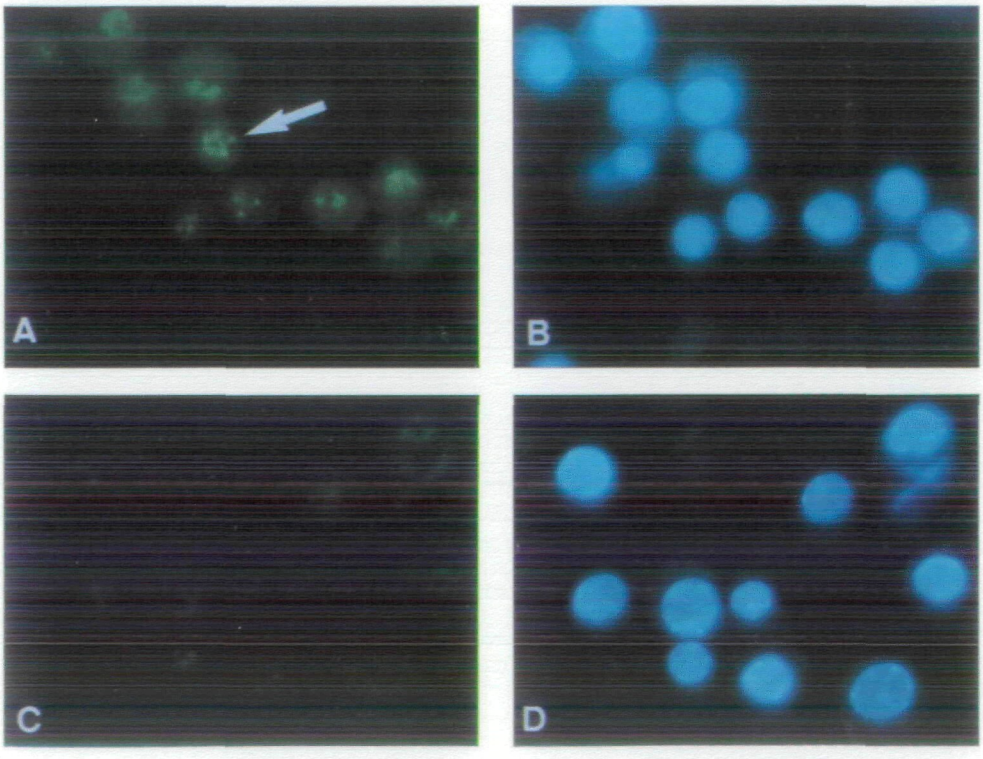

Fig. 3. **Nuclear localization of PEP.** NIH-Sape-4 cells were cultured on a coverslip, fixed with 5% acetic acid/90% ethanol and treated with an affinity-purified antibody against *Sarcophaga* PEP for 1 h followed by FITC-conjugated anti-rabbit sheep IgG solution containing DAPI. Control cells were treated with the affinity-purified antibody absorbed by recombinant PEP. (A) PEP immunofluorescence; (B) DAPI staining of (A); (C) samples treated with absorbed antibody; (D) DAPI staining of (C). The arrows indicate heterogeneous localization of PEP in the nuclei.

localized in the nuclei of growing cells and may play a role in DNA synthesis *in situ*. When Fig. 3(A) and (B) were compared carefully, it was apparent that the distribution of the PEP fluorescence was more heterogeneous than that of DAPI, as indicated by arrows, suggesting that PEP was not distributed uniformly in the nucleus, but concentrated in specific nuclear compartments. In order to investigate this further, we treated NIH-Sape-4 cells with an anti-PEP antibody followed by FITC-conjugated second antibody and propidine iodide, and examined the cells using confocal laser-scanning microscopy. As shown in Fig. 4, the distribution of the propidine iodide fluorescence, shown in red (localization of DNA), clearly differed from that of FITC, shown in green (localization of PEP). DNA was shown to be distributed throughout the nuclei, whereas PEP was localized in restricted nuclear regions where DNA was not present.

Immunoblotting of PEP—Contrary to the immunofluorescence study results, PEP was detected almost exclusively in cytoplasmic fraction when mass-isolated NIH-Sape-4 cells were fractionated into cytoplasmic and nuclear fractions and then immunoblotted (Fig. 5A). As histone was recovered only in the nuclear fraction (Fig. 5B), PEP is likely to have leaked from the nuclei during their preparation. As shown in Fig. 6, the localization of PEP in these cells clearly differed from that in the cells cultured on a coverslip (*cf*. Fig. 3). The PEP-derived fluorescence was found to be distributed throughout the cells (arrow 1), and

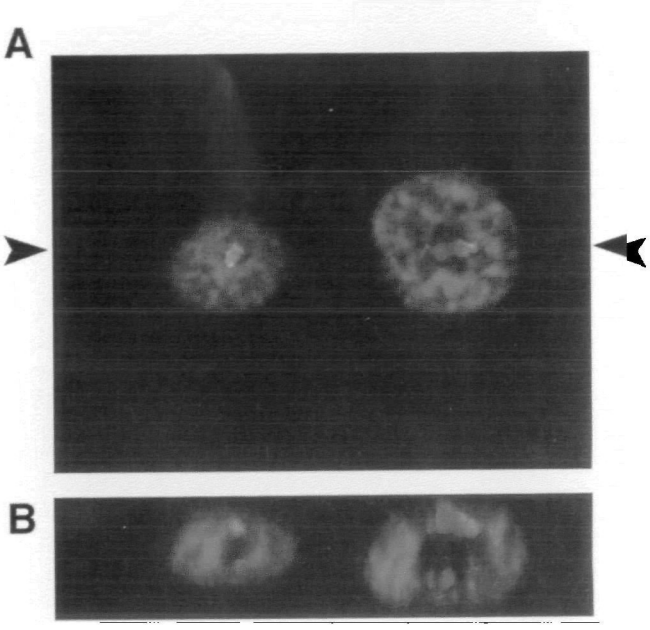

Fig. 4. **Immunofluorescence study of PEP.** Growing NIH-Sape-4 cells were treated successively with an affinity-purified antibody against *Sarcophaga* PEP and FITC-conjugated anti-rabbit sheep IgG solution containing propidine iodide and visualized by confocal laser-scanning microscopy. The localizations of PEP and DNA are shown in green and red, respectively. (A) a horizontal section; (B) a vertical section.

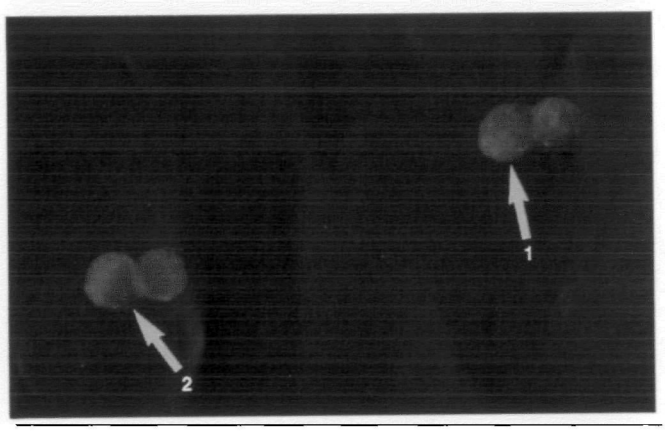

Fig. 6. **Immunofluorescence of PEP in mass-isolated cells.** Cultured cells were fixed on a slide glass and treated successively with an affinity-purified antibody against *Sarcophaga* PEP and FITC-conjugated anti-rabbit sheep IgG solution containing DAPI. Arrow 1 shows cellular distribution of PEP and arrow 2 its less dense distribution in the nuclei.

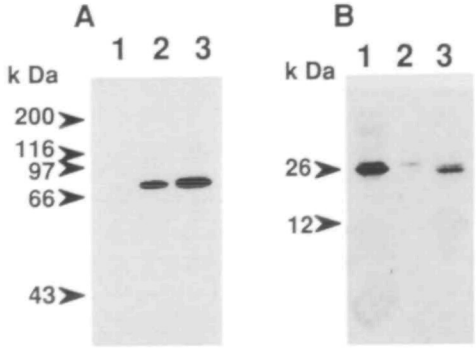

Fig. 5. **Immunoblotting of PEP.** Mass-isolated NIH-Sape-4 cells were fractionated into nuclear (1,200 × *g* precipitate) and cytoplasmic (1,200 × *g* supernatant) fractions. The total lysate, nuclear and cytoplasmic fractions (each 20 μg) were immunoblotted using 30 ng of an affinity-purified antibody against PEP (A), and an anti-histone H1 antibody (B). The gels were calibrated with various molecular mass markers, the positions of which (kDa) are shown on the left. Lysates were from: lane 1, nuclear fraction; lane 2, cytoplasmic fraction; lane 3, total lysate.

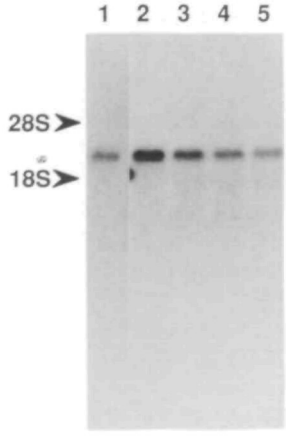

Fig. 7. **Northern blot analysis of PEP mRNA.** RNA was extracted from *Sarcophaga* embryos at various developmental stages and subjected to Northern blotting using a *Bam*HI-*Bst*1107I fragment of the coding region of *Sarcophaga* PEP cDNA. RNA was extracted from: lane 1, unfertilized eggs; lanes 2-5, embryos isolated from 6- to 9-day-old female flies.

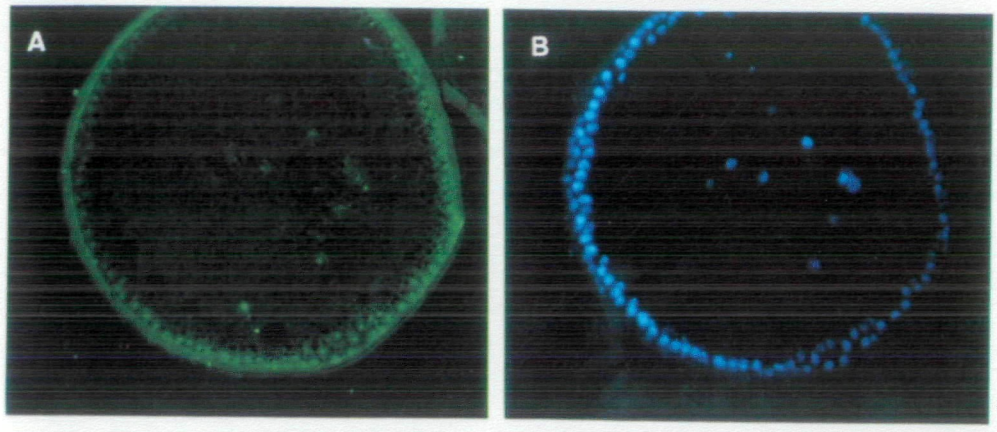

Fig. 8. **Immunofluorescence of PEP in embryos.** A section of an embryo from a 6-day-old female fly was treated successively with an affinity-purified antibody against *Sarcophaga* PEP and FITC-conjugated anti-rabbit sheep IgG solution containing DAPI. (A) PEP Immunofluorescence; (B) nuclei visualized by DAPI staining.

the nuclei of some cells were stained less densely (arrow 2). We harvested the cultured cells when they were at the stationary phase. As they grew, the cells piled up at the bottom of the culture flask and we usually shook the flask vigorously to peel the attached cells from the bottom and harvest them. These results indicate that the cellular location of PEP can be changed easily depending upon the physiological and/or environmental conditions to which NIH-Sape-4 cells are subjected.

PEP in Embryonic Development—As PEP appeared to play a crucial role in DNA synthesis, we examined PEP expression during embryogenesis by Northern blotting. As shown in Fig. 7, PEP mRNA was detected in unfertilized eggs (lane 1), indicating that a certain amount of mRNA was stored in eggs as a maternal message. The PEP gene expression level in embryos collected from 6-day-old females was significantly higher than that in unfertilized eggs and subsequently, its expression decreased gradually (lanes 2–4). As *Sarcophaga* is an ovoviviparous insect, it is difficult to synchronize the developmental stages of embryos. However, according to our experience, embryos in 6-day-old females are at a very early stage (possibly the cellular blastodermal stage) and those in 10-day-old females are at the stage just before eclosion. We examined embryos from 6-day-old females using an immunofluorescence method with an anti-PEP antibody. As shown in Fig. 8B, nuclei visualized by DAPI staining were assembled in the vicinity of the cuticle, indicating that the embryos were at the blastodermal stage, and the specific fluorescence of the anti-PEP antibody was detected almost exclusively in the nuclei (Fig. 8A), as observed with growing NIH-Sape-4 cells. The fluorescence of the outer layer was due to natural fluorescence of cuticle. Therefore, PEP appears to be essential for DNA synthesis and/or nuclear division during the embryonic development of dipteran insects.

DISCUSSION

In this study, we demonstrated that ZTTA selectively inhibited DNA synthesis by dividing insect cells. As ZTTA is a specific PEP inhibitor, our results suggest that PEP plays a crucial role in DNA synthesis and therefore, insect cell proliferation. Relatively high doses of ZTTA were needed for the inhibition of cell proliferation. We assume that this is due to low efficiency of the incorporation of ZTTA into the cells. PEP is found in various organisms, but its biological role is unknown. Therefore, this is, to the best of our knowledge, the first demonstration of a possible biological function. However, this conclusion is entirely dependent upon the specificity of ZTTA, which was demonstrated to inhibit PEP specifically when its effects on various proteinases were tested *in vitro* (*17, 18*). However, as we used cells and tissues in culture, the possibility that ZTTA interferes with an enzyme(s) other than PEP that is indispensable for DNA synthesis cannot be excluded. Therefore, this conclusion is valid only if ZTTA is proven to be a specific PEP inhibitor under our conditions.

Hitherto, nothing was known about the cellular localization of PEP. We used a confocal laser-scanning microscopy and demonstrated that PEP was localized almost exclusively in restricted regions of the nuclei of growing NIH-Sape-4 cells. When these cells are treated with propidine iodide, DNA in the nuclei is stained, but nucleoli should remain unstained. When Fig. 4 was examined carefully, nucleoli-like structures that were not stained by propidine iodide could be seen and fluorescence due to PEP seems to be limited to areas near these structures. If PEP is needed for DNA synthesis, it would be expected to be localized in all the areas where DNA is present. However, as each image of Fig. 4 represents only one cross-sectional plane, it is difficult to speculate from these images what the spatial distribution of PEP in a nucleus is, and thus, the mechanism responsible for its participation in DNA synthesis *in situ*. Nuclear localization of PEP was also found to be localized in the nuclei of *Sarcophaga* embryos.

The immunofluorescence and immunoblotting results are rather contradictory. As we used growing cells for the former experiments and cells at stationary phase for the latter, we assume that PEP leaks from and reassembles in nuclei readily depending upon the cellular physiological conditions. Otherwise, it is difficult to interpret these results. However, unlike SV40 large T-antigen (*25*) or nucleoplasmin (*26*), no known nuclear localization signals were detected in the *Sarcophaga* PEP sequence.

It is noteworthy that PEP mRNA was found to be stored as a maternal message in unfertilized eggs. During the embryonic development of dipteran insect, division and migration of nuclei proceed prior to cell formation. Perhaps, maternal PEP mRNA is translated at a very early stage of embryonic development and the resulting PEP participates in DNA synthesis and/or nuclear division at the stage of syncytial stage.

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