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Hydrolysis and Synthesis of Substrate Proteins for Cathepsin L in the Brain Basement Membranes of *Sarcophaga* during Metamorphosis[1]

Ikuko Fujii-Taira,*,† Yasuhiro Tanaka,*,† Koichi J. Homma,† and Shunji Natori*,[2]

Natori Industry-Sponsored Laboratory, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-0198; and †Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033

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Previously, we identified two proteins with molecular masses of 200 and 210 kDa in basement membranes of *Sarcophaga* imaginal discs as substrates for cathepsin L [Homma, K. and Natori, S. (1996) *Eur. J. Biochem.* 240, 443–447]. Here we demonstrated that the same proteins were also present in the basement membranes of larval brains. These proteins were suggested to be digested by cathepsin L secreted from the larval brains in response to 20-HE. From the behavior of these proteins during metamorphosis, we concluded that the basement membranes of larval brains are degraded at the early pupal stage and synthesized again at the late pupal stage, coinciding with the timing of brain remodeling that takes place during metamorphosis. Possibly, the transient disappearance of the basement membranes makes brain remodeling easier, and cathepsin L is suggested to play a crucial role in the degradation of the basement membranes.

Key words: basement membrane, cathepsin L, 20-hydroxyecdysone, neural remodeling, *Sarcophaga peregrina*.

The metamorphosis of holometabolous insects is a striking event accompanied by extensive tissue alteration and remodeling. Namely, most larval tissues are decomposed and adult tissues are newly constructed from imaginal discs during metamorphosis. Imaginal discs differentiate into adult structures under the control of ecdysone (*1*, *2*). Previously, we demonstrated that imaginal discs of *Sarcophaga peregrina* (flesh fly) secreted cathepsin L in the presence of 20-hydroxyecdysone (20-HE) and this proteinase was implicated in the differentiation of imaginal discs (*3*). Cathepsin L is usually a lysosomal enzyme, but its sorting process seems to change under the control of 20-HE and it becomes a secretory protein under these conditions. We found that cathepsin L digested the basement membranes of imaginal discs in an autocrine manner, which makes it possible for imaginal discs to extend spatially. Furthermore, we identified two proteins in the basement membranes of imaginal discs with molecular masses of 200 and 210 kDa, which are assumed to be substrates for cathepsin L (*4*).

Metamorphic changes of the central nervous system (CNS) are also induced by 20-HE during metamorphosis. Some larval neurons undergo apoptosis and new neurons develop from neuroblasts. This extensive remodeling of the larval CNS results in construction of the adult CNS (*5*, *6*). We succeeded in reproducing morphological changes of the larval CNS that take place at the early pupal stage (*7*). This was achieved by culturing larval brains *in vitro* in the presence of 20-HE. These changes mainly consist of enlargement of the brainhemispheres and extension of the interval between the two hemispheres. As cathepsin L has been suggested to be an important proteinase that digests the basement membranes of imaginal discs when they differentiate into adult structures, we examined whether or not the same enzyme participates in remodeling of the CNS using an *in vitro* culture system.

Here we report that the basement membranes of larval brains contain the two substrate proteins for cathepsin L, and that they are digested during metamorphosis, suggesting that cathepsin L plays a role in brain remodeling as well as in imaginal disc development in *Sarcophaga*.

MATERIALS AND METHODS

In Vitro Culture of Sarcophaga Brains—Larval brains were dissected out and cultured essentially as described previously (*7*). Briefly, brains with eye-antenna discs, prepared from third instar larvae, were cultured in 350–500 μl of Grace's insect medium at 25°C under a humidified atmosphere. The medium was modified before use by adding HEPES to a final concentration of 10 mM, adjusting the pH to 6.0 with 1 M NaOH, and adding final concentrations of 100 units/ml of penicillin G and 0.5 mg/ml of streptomycin. To induce metamorphic changes of the brains, 20-HE was added to the medium at a final concentration of 10^{-6} M. To prepare brains without eye-antenna discs, the eye-antenna discs were severed from brains at the optic stalks with a fine needle.

Antibodies—Polyclonal antibodies against *Sarcophaga*

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[2] To whom correspondence should be addressed. Tel: +81-48-467-9437, Fax: +81-48-462-4693

Abbreviations: 20-HE, 20-hydroxyecdysone; CNS, central nervous system; PBS, phosphate-buffered saline; v/v, volume per volume; w/v, weight per volume; FITC, fluoresceinisothiocyanate; TBS, Tris-buffered saline.

pro-cathepsin L and the two basement membrane proteins with molecular masses of 200 and 210 kDa were used throughout. The preparation and specificity of these antibodies were reported elsewhere (*3*, *4*). Antibody against pro-cathepsin L was referred to as anti-cathepsin L antibody.

Immunofluorescence Study—Brains were fixed in 10 mM phosphate-buffered saline (PBS) supplemented with Carnoy's fixative (ethanol/chloroform/acetic acid, 6:3:1, v/v) for 30 min, and then washed with PBS containing 0.5% (v/v) Tween 20. The brains were then successively treated with 50 μl of an affinity-purified antibody solution (10 μg/ml) and 25 μl of swine anti-rabbit IgG conjugated with FITC for 1 h each at room temperature. After immersing the brains in PBS containing 2.5% (w/v) 1,4-diazabicyclo-[2,2,2]octane for 5 min, they were finally immersed in 90% (v/v) fluorescence-free glycerol in the same PBS, and mounted on coverslips for examination. The fluorescence of FITC was visualized by confocal microscopy.

Western Blotting—Samples were treated with 10% TCA and acid-insoluble materials were subjected to SDS polyacrylamide gel electrophoresis (*8*). The separated proteins were transferred electrophoretically from the gel to polyvinylidene difluoride membrane filters. The filters were immersed in 5% skim milk in TBS-T (10 mM Tris/HCl, pH 7.9, containing 150 mM NaCl and 0.1% Triton-X 100) for 1 h. Then the filters were transferred to a rinse solution (0.25% skim milk in TBS-T) containing 1 mM EDTA and 30–90 ng/ml of affinity-purified antibodies, and kept for 8–12 h at 4°C. They were then washed with the rinse solution, transferred to 5 ml of the rinse solution containing radioiodinated swine anti-rabbit IgG (18.5 kBq), and kept for 2 h at room temperature. Finally, the filters were washed thoroughly with the rinse solution, dried, and subjected to autoradiography using Kodak XAR film. The samples examined were culture medium and brain lysates. Brain lysates were prepared in 10 mM phosphate buffer, pH 7.2, containing 10 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 1 mM phenylmethanesulfonyl fluoride.

RESULTS

Detection of Substrate Proteins for Cathepsin L in Sarcophaga Larval Brains—Previously, we identified two proteins with molecular masses of 200 and 210 kDa that were readily digested by cathepsin L in the basement membranes of leg imaginal discs (*4*). Digestion of these proteins was assumed to be essential for extension of the imaginal discs during metamorphosis. We performed an immunofluorescence study to determine whether or not the same proteins are present in the basement membranes of larval brains. Brains were successively treated with antibody and FITC-conjugated secondary IgG, and then visualized by confocal microscopy. As shown in Fig. 1, the fluorescence of both proteins was detected on the surface, but not inside, of the brainhemispheres. Like leg discs, the surface of eye-antenna discs attached to the brainhemispheres was also stained. Fluorescence was also detected on the surface of peripheral nerves. These results suggest that the 200 and 210 kDa proteins are ubiquitously present in the basement membranes of various larval tissues.

Secretion of Cathepsin L from Larval Brains—As reported previously, we found that larval brains change morphologically when cultured *in vitro* in the presence of 20-HE, the morphological changes that occur during metamorphosis being mimicked (*7*). For these morphological changes, the basement membranes may have to be digested. As the two substrate proteins for cathepsin L were detected in the basement membranes of brains, we examined whether or not larval brains secrete cathepsin L when cultured in the presence of 20-HE. As imaginal discs were shown to secrete cathepsin L under these conditions (*3*), we carefully removed the eye-antenna discs from the brainhemispheres and cultured the brains in the presence or

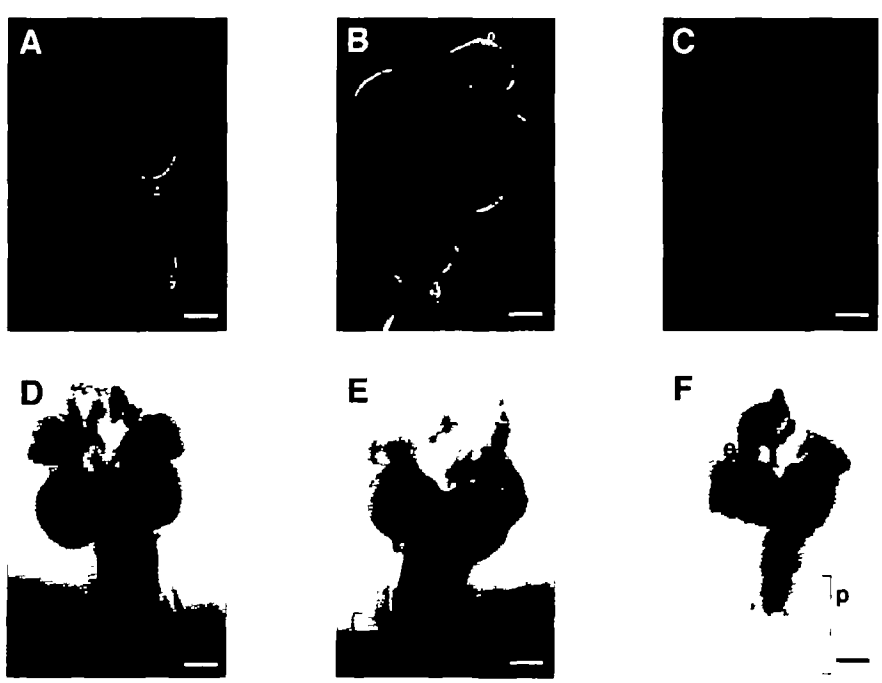

Fig. 1. **Immunofluorescence study of the 210- and 200-kDa proteins in brains.** Brains from third instar larvae were subjected to immunofluorescence staining with affinity-purified antibodies against the 200 and 210 kDa proteins, and visualized by confocal microscopy. (A) Immunofluorescence of the 200-kDa protein; (B) Immunofluorescence of the 210-kDa protein; (C) Control with normal antibodies; (D), (E), and (F) are bright fields of (A), (B), and (C), respectively. b, brainhemisphere; e, eye-antenna disc; p, peripheral nerves. The bars indicate 200 μm.

absence of 10^{-6} M 20-HE. Then the proteins in the culture medium were subjected to Western blotting using anti–cathepsin L antibody. As shown in Fig. 2, signals were detected for the culture medium containing 20-HE, indicating that brains secrete cathepsin L in the presence of 20-HE, like imaginal discs. Moreover, the medium recovered after 2 days contained only pro-cathepsin L (50 kDa), but that recovered after 4 days contained mature cathepsin L (35 kDa). These results indicate that the brains first secrete pro-cathepsin L, which is eventually processed into mature cathepsin L. Thus, it was suggested that cathepsin L secreted from brains in the presence of 20-HE digests their basement membranes, and this process is essential for enlargement of the brainhemispheres and extension of the interval between the two hemispheres.

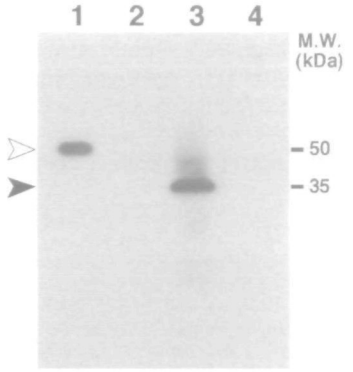

Fig. 2. **Western blotting of cathepsin L.** Brains without eye-antenna discs were cultured in the presence or absence of 20-HE. Culture medium was collected and proteins in it were subjected to Western blotting using affinity-purified antibody against pro-cathepsin L. Culture medium for one brain was applied to each lane. Sources of medium: lanes 1 and 3, medium cultured with 20-HE for 2 and 4 days, respectively; lanes 2 and 4, medium cultured without 20-HE for 2 and 4 days, respectively. The positions of pro-cathepsin L and mature cathepsin L are indicated by open and closed arrowheads, respectively.

Digestion of the 200 and 210 kDa Proteins in Brain Basement Membranes—Next, we performed Western blotting of larval brain lysates to determine whether or not the two basement membrane proteins are digested on incubation of larval brains with 20-HE (Fig. 3). For this, we first incubated larval brains in the presence of 10^{-6} M 20-HE for 1–3 days. Then we removed the eye-antenna discs to prevent contamination of the disc basement membranes and prepared brain lysates using the resulting brainhemispheres. The intensity of the bands of the 200 and 210 kDa proteins tended to decrease with time. In addition to the 200 kDa protein, three proteins with molecular masses of 175, 110, and 100 kDa were detected with anti–200-kDa protein antibody, and the intensity of the bands of the latter two proteins increased with time (Fig. 3A). When probed with anti–210-kDa protein antibody, a protein with a molecular mass of 120 kDa was detected in addition to the 210 kDa protein, and the intensity of the band of the 120 kDa protein also increased (Fig. 3B). Possibly, the extra proteins

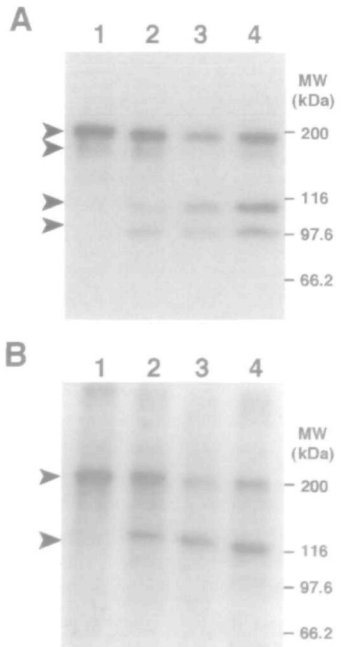

Fig. 3. **Detection of the 210 and 200 kDa proteins in larval brains and their breakdown in the presence of 20-HE.** Brains with eye-antenna discs were cultured in the presence or absence of 20-HE for 1–3 days. Then the eye-antenna discs were removed and brain lysates (20 μg protein in each lane) were subjected to Western blotting using affinity-purified antibodies against the 200-kDa protein (A) and the 210-kDa protein (B). Lane 1, intact brains; lanes 2–4, brains cultured with 20-HE for 1, 2, and 3 days, respectively. The arrowheads indicate the proteins that reacted with the respective antibodies.

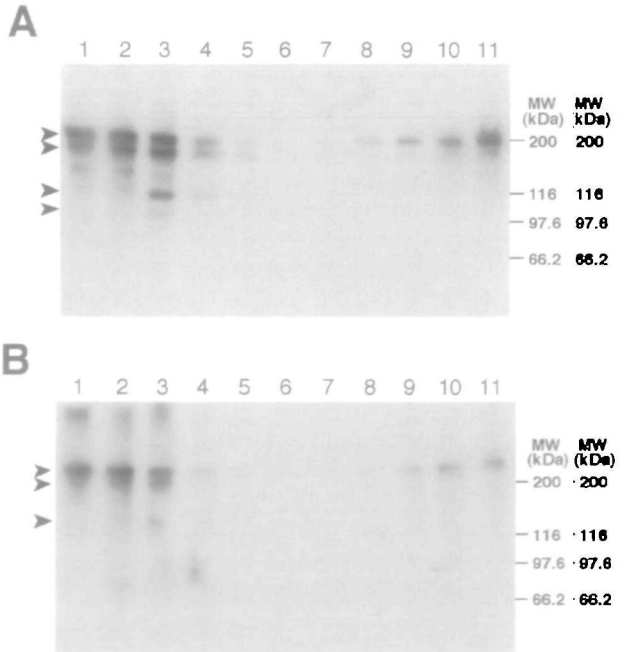

Fig. 4. **Disappearance and reappearance of the two basement membranes proteins during metamorphosis.** Brains were collected on the indicated days after pupation, and then lysates of them (40 μg protein in each lane) were subjected to Western blotting using affinity-purified antibodies against the 200-kDa protein (A) and the 210-kDa protein (B). Pupae on day 0 are equivalent to third instar larvae and those on day 10 are equivalent to adults. Lanes 1–11, brains from pupae on days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 after pupation, respectively. The arrowheads indicate the proteins that reacted with the respective antibodies.

detected with each antibody are degradation products of the 210 or 200 kDa protein. They are likely to be produced by digestion of the original proteins by cathepsin L secreted from the brains. These patterns were very similar to those obtained with leg discs incubated in the presence of 20-HE (*4*).

Disappearance and Reappearance of the Basement Membrane Proteins during Metamorphosis—The basement membranes of brains were suggested to be degraded during brain remodeling reproduced *in vitro*, as revealed by the decreases in the 210 and 200 kDa proteins. We examined if the same thing happens *in vivo*. We isolated the brains of pupae at various developmental stages, and prepared lysates and subjected them to Western blotting. As shown in Fig. 4, much of both the 200 and 210 kDa proteins had disappeared by day 4, and appeared again on day 8 after pupation. The pupal stage of *Sarcophaga* lasted for 10 days under our rearing conditions. Thus, pupae on day 10 after pupation were equivalent to adult flies. These results suggested that the basement membranes of larval brains are digested at the early pupal stage and synthesized again when brain remodeling has been completed at the late pupal stage. Possibly, the 200 and 210 kDa proteins are digested by cathepsin L, since several degradation products obtained on their digestion with cathepsin L were detected in brains at the early pupal stage. These proteins seem to be synthesized *de novo* and the basement membranes are formed again during the last 3 days of the pupal stage.

DISCUSSION

During the metamorphosis of holometabolous insects, larval tissues are decomposed and adult tissues are constructed *de novo* from imaginal discs. Previously, we demonstrated that the basement membranes of *Sarcophaga* imaginal discs are digested by self-secreted cathepsin L, and that this process is essential for their spatial extension during metamorphosis (*3*). We identified two proteins (200 and 210 kDa proteins) in the basement membranes of imaginal discs as substrates for cathepsin L (*4*). It became evident that the basement membranes of *Sarcophaga* larval brains are probably the same as those of imaginal discs in terms of the presence of these two proteins.

We demonstrated that, like imaginal discs, larval brains secreted cathepsin L on treatment with 20-HE. Both imaginal discs and brains are known to undergo morphological changes during metamorphosis. However, their changes are principally different. Imaginal discs develop into adult structures that are totally different from the original imaginal discs. Contrary to imaginal discs, brain structures remain almost as they are during metamorphosis, but larval neurons are partly replaced by adult ones, which is called brain remodeling.

Previously, we demonstrated that enlargement of the brainhemispheres and extension of the interval between the two hemispheres occurred at the early pupal stage (*7*). As the 200 and 210 kDa proteins disappear at this stage, we suspect that the basement membranes of brains are degraded under these conditions, although the overall brain structures do not change significantly, and that disappearance of the basement membranes is a prerequisite for brain remodeling. It is known that extracellular matrices, including basement membranes, participate in the formation of neural networks, such as in axon guidance and axon regeneration (*9–11*). Proteolytic modulation of extracellular matrix proteins sometimes promotes cell adhesion and cell migration (*12–15*). Therefore, degradation of the 200 and 210 kDa proteins itself might make sense for the reorganization of neural networks.

We found that these two proteins appear again at the late pupal stage. Possibly, these proteins are synthesized in brains to construct basement membranes again, suggesting that the composition of the basement membranes of adult brains is the same as that of larval brains. It is likely that the syntheses of these proteins are controlled at the transcription level, but nothing is known about the regulatory mechanism for this.

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