Short Communication

Generation and Characterization of Monoclonal Antibodies Specific to *Drosophila presenilin*

Ho Thi Thu Cuc,^{1,*} Jong Bok Seo,^{2,*} Jin Kyu Choi,¹ Won Tae Kim,^{1,3} Seok Jou Park,¹
Dae Weon Lee,¹ Yong Sun Kim,¹ Mark E. Fortini,⁴ and Young Ho Koh¹

The development of a monoclonal antibody (MAb) specific to *Drosophila presenilin* (*Psn*) proteins *in vivo* was the major aim of this study, since the absence of specific antibodies recognizing *Psn* proteins hampered our progress in understanding *Psn* functions during development, differentiation, and pathogenesis. By dot blot and immunofluorescence screenings, we found that MAb Psn2G6 specifically recognized *Psn* proteins in wing imaginal discs and brains of wild-type control *W1118* larvae. MAb Psn2G6 also transgenically expressed a long form of wild-type *Psn* (*Psn* + 14 WT) proteins in wing imaginal discs of two independent transgenic lines. Transgenic expression of *Psn* + 14 WT proteins in *psn*^{B3} larvae completely rescued the expression patterns of *Psn* proteins and the development of wing imaginal discs. In addition, neural hyperplasia observed in wing imaginal discs of *psn*^{B3} larvae was also suppressed.

Introduction

ALZHEIMER'S DISEASE (AD) is the most common progressive neurological disorder in the elderly population. Since the progression and the pathological hallmarks of rare familial AD (FAD) share a high degree of similarity with common sporadic AD (SAD), studies in FAD have contributed to our understanding of the molecular genetics, biochemistry, and pathophysiology underlying SAD.^(1,2) Presenilins are a family of polytopic transmembrane proteins that function as a part of the γ -secretase protease complex.^(3,4) Mutations in human presenilin-1 (PS1) and PS2 are linked to pathology of FAD in two ways. One is that FAD-linked mutations in PSs accelerate accumulation of more toxic amyloid β -42 peptide in amyloid plaques in brain tissues.⁽⁵⁾ Another pathway that FAD mutations in PSs are associated with in AD pathology involves a partial loss of PS activities.⁽⁶⁾ Reduced PS activities may be responsible for progressive memory and cognitive decline and neurodegeneration in brains through amyloid β -42-independent pathways.

The *Drosophila presenilin* (*Psn*) amino acid sequence is 50% identical to human PS1 and PS2.⁽⁷⁾ Two alternatively spliced variants of *Drosophila Psn* encoding 527 and 541 amino acid polypeptides are reported. The long form of *Psn* is named *Psn* + 14 WT, since a 14 amino acid insertion within the large loop domain is present and has been shown to rescue *psn* null mutant phenotypes when it was transgenically expressed under control of a *presenilin* endogenous promoter cassette (PEPC).⁽⁸⁾ *Drosophila psn* mutant animals lacking endogenous *Psn* activities cannot complete the larval-pupal transition during morphogenesis.^(9,10) Introduction of FAD-linked mutations in PSs into *Drosophila Psn* allows us to assess the genetic properties of human PS FAD mutations.⁽⁸⁾ Thus, *Drosophila* can be used as a genetic model to study the molecular and cellular etiologies underlying FAD. However, the absence of specific antibodies to *Psn* hampers our progress in understanding functions of *Psn* during development and FAD pathogenesis.

In this study, we generated and characterized a monoclonal antibody (MAb) that specifically recognized endogenous *Psn*

¹Ilson Institute of Life Science, Hallym University, Dongan-gu, Anyang, Gyeonggi-do, Republic of Korea.

²Metabolome Research Team, Seoul Branch, Korea Basic Science Institute, Sungbuk-Gu, Seoul, Republic of Korea.

³National Institute of Agricultural Science and Technology, Seodeun-dong, Gwonseon-gu, Suwon, Gyeonggi-do, Republic of Korea.

⁴Department of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania.

*These authors contributed equally to this work.

proteins as well as transgenically expressed *Psn* + 14 WT proteins *in vivo*.

Materials and Methods

Mice

Female Balb/c mice were obtained from Central Laboratory Animal (Seoul, Korea). All experimental procedures for this study were performed according to the guidelines of the Animal Ethics Committee of Hallym University (Chuncheon, Korea).

Drosophila strains

Previously characterized *W1118*, *psn*^{B3}/*TM6C*, *cu*¹ *Sb*¹ *Tb*¹, *psn*^{C4}/*TM6C*, *cu*¹ *Sb*¹ *Tb*¹, *psn*^{S3}/*TM6C*, *cu*¹ *Sb*¹ *Tb*¹, *psn*^{I2}/*TM6C*, *cu*¹ *Sb*¹ *Tb*¹, PEPC (*presenilin* endogenous promoter cassette)-*Psn* + 14 WT, and *psn*^{B3} line 1 and line 8^(8,11) were employed for this study. *W1118* was a wild-type control animal for the experiments. *psn*^{B3} mutation is known to have a single amino acid substitution (G516E) near the C-terminal tail of *Psn* protein (Fig. 1A). *psn*^{C4}, *psn*^{S3}, and *psn*^{I2} each have a point mutation replacing a tryptophan codon with a stop codon (W152Stop, W237Stop, and W278Stop, respectively) before the *Psn* loop domain (Fig. 1A).⁽¹¹⁾ PEPC-*Psn* + 14 WT and *psn*^{B3} lines 1 and 8 are two independent transgenic lines expressing the long form of wild-type *Psn* protein in a *psn*^{B3} mutant background.⁽⁸⁾

Antigens

A loop peptide sequence (NH₂-(C)QRTGNSHPRQNQRD DGSVLA-COOH, amino acid 362–381) from the *Drosophila* *Psn* protein was synthesized using a 431A peptide synthesizer (Applied Biosystems, Foster City, CA) in the Organic Chemistry Laboratory of Ilsong Institute of Life Sciences, Hallym University. All peptides were purified by FPLC using a C₁₈ column on an AKTA purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). Purified peptides were conjugated to keyhole limpet hemocyanin (KLH, Pepton Inc., Daejeon, Korea) for use as immunogens.

Generation of monoclonal antibody

The detailed procedure for generating hybridoma clones producing MABs is described in a previous study.⁽¹²⁾ Briefly, Balb/c mice were immunized intraperitoneally with 50 mg of KLH conjugated peptides in complete Freund's adjuvant

(Thermo Fisher Scientific, Waltham, MA). Three immunized mice were boosted four times intraperitoneally with the same dose of antigens in incomplete Freund's adjuvant (Thermo Fisher Scientific) with a 2-week interval before somatic fusion. Spleen cells acquired from immunized mice 2 days after the last boost were fused with mouse myeloma cell line Sp2/0 by using polyethylene glycol 1500 (Roche, Basel, Switzerland) and then were screened in hypoxanthine-aminopterin-thymidine (HAT) medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mM HEPES buffer, 1x HAT supplement, and antibiotics). 1032 hybridoma clones were generated from three immunized mice.

Dot blot screenings

Purified *Psn* peptides were used to screen 1032 hybridoma clones to identify those clones producing antibodies specific to immunogens. A 96-well dot blot apparatus (Bio-Rad, Hercules, CA) was set up with nitrocellulose (NC) membrane (Amersham Pharmacia Biotech), and each dot was rinsed with 200 mL of 0.1 M phosphate-buffered saline with 0.05% Tween-20 (PBST). Under vacuum, 100 ng of purified peptides in PBST were added in each dot and then rinsed three times with 200 mL of PBST. NC membranes were blocked with 3% non-fat dried milk in PBST for 1 h with shaking at room temperature (RT). 200 mL of supernatants of 1032 hybridoma clones were added into each dot and then incubated overnight at 4°C with shaking. After washing three times with PBST, membranes were blocked with 3% non-fat dried milk in PBST for 1 h. Membranes were incubated in 1:5000 diluted HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific) for 1 h at RT with shaking. After additional washing with PBST, the membranes were treated with an enhanced chemi-fluorescence agent (Thermo Fisher Scientific). 132 hybridoma clones secreted antibodies recognizing immunogens. 132 hybridoma clones were further analyzed for immunofluorescence analysis.

Psn MAB immunofluorescence screenings and immunofluorescence microscopy

Third instar wandering stage larvae were dissected in Ca²⁺ free saline (128 mM NaCl, 2 mM KCl, 4 mM MgCl₂·6H₂O, 35.5 mM sucrose, 5 mM HEPES, 1 mM EGTA) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2).

FIG. 1. MAB Psn2G6 revealed specific localization patterns of *Psn* proteins in the wing imaginal discs in *W1118* larvae. To examine the specificity of MAB Psn2G6, four *psn* mutant alleles were employed (A). Predicted amino acid substitutions in four different *psn* mutant alleles are depicted at the proposed structure and topology of the *Psn* proteins (modified from Lukinova et al.¹¹ and Spasic et al.⁴). Transmembrane domains 1 to 9 (T1 to T9) are indicated by green barrels. MAB Psn2G6 strongly recognized *Psn* proteins in wing imaginal discs in *W1118* larvae (B1). Strong MAB Psn2G6 immunoreactivity localized to surface folds in wing imaginal discs. Even though wing imaginal discs of *psn*^{B3} larvae did not develop, the immunoreactivity of MAB Psn2G6 in *psn*^{B3} (C1) was stronger than those in *psn*^{C4}/*psn*^{S3} (D1) or *psn*^{S3}/*psn*^{I2} (E1). Rabbit polyclonal Fasciclin II (FasII) antibodies were used to examine the gross morphology of wing imaginal discs of *W1118* (B2), *psn*^{B3} (C2), *psn*^{C4}/*psn*^{S3} (D2), and *psn*^{S3}/*psn*^{I2} (E2). MAB Psn2G6 immunoreactivity partially co-localized with FasII signals in *W1118* (B3) and *psn*^{B3} (C3). When wing imaginal discs were incubated with cell culture media, very weak signals were detected from all wing imaginal discs (B1–E1). The fate map of wing imaginal discs^(14,15) was used to investigate expression patterns of *Psn* proteins labeled by MAB Psn2G6 (F). Strong signals were present PWP, PVR, MCo, DCo, PDR, AS1, ANWP, PNWP, AC, and AL. *Psn* proteins in neurophil in VNS and OLs in CNS were labeled by MAB Psn2G6 (G). A, P, D, V, presumptive anterior, posterior, dorsal, ventral surface of adult wings; ANWP, anterior notal wing process; PNWP, posterior notal wing process; AC, axillary cord; AL, alar lobe; AS1, axillary sclerites1; DCo, distal costa; MCo, medial costa; PCo, proximal costa; PDR, proximal dorsal radius; PVR, proximal ventral radius; PWP, pleural wing process; CNS, central nervous system; OL, optic lobe; VNS, ventral nervous system; bar = 100 mm.

After washing three times with 0.1 M phosphate buffer with 0.2% Triton-X 100 (PBT), dissected larvae were incubated with 132 MAbs and various other antibodies. Rat-MAb-Elav-7E8A10⁽¹³⁾ obtained from the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA) was used to label neural hyperplasia in wing imaginal discs. Polyclonal rabbit anti-

Fascilin II antibodies⁽¹⁴⁾ were used to stain epithelial cell junctions in wing imaginal discs. Goat anti-mouse IgG-Alexa 488, goat anti-mouse IgG-Alexa 568, goat anti-rat-Alexa 488, and donkey anti-rabbit IgG-Alexa 568 (Invitrogen, Carlsbad, CA) were used as secondary antibodies. A confocal laser scanning microscope (LSM 510, Carl Zeiss MicroImaging,

FIG. 2. MAb Psn2G6 recognizes transgenically expressed *Psn* + 14 WT proteins, and neural hyperplasia in wing imaginal discs in *psn*^{B3} mutant larvae are rescued by transgenic expression of *Psn* + 14 WT proteins. *Psn* immunoreactivity was observed in wing imaginal discs of W1118 (A), *psn*^{B3} (B) *PEPC-Psn* + 14 WT; *psn*^{B3} line 1 (C), and *PEPC-Psn* + 14 WT; *psn*^{B3} line 8 (D). Transgenic expression of *Psn* + 14 WT proteins completely rescued the abnormal development of wing imaginal discs in *psn*^{B3} mutant animals. Wing imaginal discs of W1118 larvae did not show any rat MAb-Elav-7E8A10 signals (E). However, wing imaginal discs in *psn*^{B3} mutant larvae showed strong signals, indicated by arrow (F). Transgenic expression of *Psn* + 14 WT proteins in wing imaginal discs rescued neural hyperplasia phenotypes, even though several cells in a wing imaginal disc in *PEPC-Psn* + 14 WT; *psn*^{B3} line 1 were labeled (G). MAb-Elav-positive cells are indicated by arrowheads. In contrast, there was no MAb-Elav-positive cell in a wing imaginal disc of *PEPC-Psn* + 14 WT; *psn*^{B3} line 8 (H); bar = 100 μ m.

Oberkochen, Germany) was used to obtain digital images of wing imaginal discs and central and ventral nervous systems. To examine the background staining from second antibodies, W1118, *psn*^{B3}, *psn*^{C4}/*psn*^{S3}, and *psn*^{S3}/*psn*^{I2} were incubated with cell culture media (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mM HEPES buffer, and antibiotics). Samples were labeled with second antibodies and then digital images of imaginal discs were obtained by LSM510. LSM image browser (Carl Zeiss MicroImaging), Image J (Scion Corporation, Frederick, MD), and Adobe Photoshop (Adobe Systems, San Jose, CA) were used for processing images.

Results and Discussion

Generation of hybridoma clones secreting *Drosophila* Psn protein-specific MAbs

Since MAbs specific to *Drosophila* Psn proteins were not available, the major goal of this study was to generate Psn-specific MAbs and examine the precise localization patterns of Psn *in vivo*. To accomplish this goal, we screened 1032 hybridoma clones from three immunized mice by dot blot assays. Supernatants of 132 hybridoma clones recognized purified Psn peptides (data not shown). It has been shown that Psn is widely expressed throughout

oogenesis, embryogenesis, and imaginal disc development.⁽⁷⁾

To identify specific clones secreting MABs for *Psn*, MABs from 132 hybridoma clones were screened by performing immunofluorescence microscopy. Among the 132 hybridoma clones tested, MAB Psn2G6 showed the most strong and specific *Psn* immunoreactivity in wing imaginal discs of *W1118* larvae (Fig. 1B1). *Psn* immunoreactivity localized predominantly at the apical membranes of wing imaginal discs. Surface folds in wing imaginal discs were prominently stained. To verify the specificity of MAB Psn2G6, wing imaginal discs of *psn*^{B3}, *psn*^{C4}/*psn*^{S3}, and *psn*^{S3}/*psn*^{I2} were examined (Fig. 1C1–1E1). *psn*^{C4}, *psn*^{S3}, and *psn*^{I2} each bear a point mutation replacing a tryptophan codon with a termination codon, and *psn*^{B3} has a single amino acid replacement (Fig. 1A).⁽¹¹⁾ The *psn*^{B3}, *psn*^{C4}, *psn*^{S4}, and *psn*^{I2} mutants fail to develop normal imaginal discs, resulting in death during metamorphosis.^(9–11) Since A loop peptide sequence (NH₂-(C)QRTGNSHPRQNQRDDGSVLA-COOH, amino acid 362–381) from the *Psn* proteins was used as the antigen, predicted truncated *Psn* proteins present in *psn*^{C4}/*psn*^{S3} and *psn*^{S3}/*psn*^{I2} larvae should not be detected by MAB Psn2G6.

The MAB Psn2G6 immunoreactivity in wing imaginal discs of *psn*^{C4}/*psn*^{S3} and *psn*^{S3}/*psn*^{I2} appeared to be weaker than those of *psn*^{B3} or *W1118*. When cell culture media (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mM HEPES buffer, and antibiotics) were used instead of MAB Psn2G6, weak signals were observed from all examined genotypes (Fig. 1B4–1E4). Those weak signals were similar to those observed from wing imaginal discs in *psn*^{C4}/*psn*^{S3} and *psn*^{S3}/*psn*^{I2} stained with MAB Psn2G6 (Fig. 1D1 and 1E1). This result suggested that immunoreactivity observed from wing imaginal discs of *psn*^{C4}/*psn*^{S3} and *psn*^{S3}/*psn*^{I2} labeled with MAB Psn2G6 were only background staining.

Rabbit polyclonal anti-Fasciclin II antibodies were used to stain epithelial cell junctions in wing imaginal discs. Fasciclin II is a *Drosophila* homologue of cell adhesion molecules expressed in imaginal discs throughout development.⁽¹⁵⁾ Cellular junctions in *W1118*, *psn*^{B3}, *psn*^{C4}/*psn*^{S3}, and *psn*^{S3}/*psn*^{I2} were labeled by rabbit polyclonal anti-Fasciclin II (FasII) antibodies (Fig. 1B2–1E2).

Comparison of fate map and expression pattern of *Psn* proteins in wing imaginal discs

To understand expression patterns of *Psn* proteins in wing imaginal discs, we compared patterns of MAB Psn2G6 immunoreactivity with the wing imaginal disc fate map^(16,17) (Fig. 1F). Strong MAB Psn2G6 signals were detected in regions corresponding to the pleural wing process (PWP), proximal ventral radius (PVR), medial costa (MCo), distal costa (DCo), alar lobe (AL), axillary cord (AC), and the boundaries between axillary sclerites 1 (AS1) and anterior or posterior notal wing process (ANWP or PNWP). In contrast, the ventral and dorsal wing surface, notum, scutellum, and tegula showed weak immunoreactivity.

Psn proteins were expressed in larval brains

In addition to imaginal discs, MAB Psn2G6 recognized *Psn* proteins in larval brains (Fig. 1F). *Psn* immunoreactivity was localized at optic lobes (OL) in central nervous systems (CNS) and neurophil regions in ventral nerve cord (VNS). This result

suggested that *Psn* may play important roles in synaptic transmission and plasticity in *Drosophila* larvae. Indeed, it has been shown that mutations in *psn* induced abnormal development of synaptic structures and functions and impaired learning and memory.^(18,19)

MAB Psn2G6 recognized transgenically expressed *Psn* + 14 WT proteins

To further confirm the specificity of MAB Psn2G6, we examined whether transgenically expressed *Psn* + 14 WT proteins in *psn*^{B3} mutant animals can be detected by MAB Psn2G6. Two independent lines expressing *Psn* + 14 WT under the control of *PEPC* (*presenilin endogenous promoter cassette*) in *psn*^{B3} mutant background were examined. Transgenically expressed *Psn* + 14 WT proteins in *psn*^{B3} were labeled by MAB Psn2G6 and completely rescued abnormal development of wing imaginal discs (Fig. 2C and 2D). Expression patterns *Psn* in wing imaginal discs of two transgenic lines were comparable to those of *W1118* (Fig. 2A, 2C, and 2D). However, *psn*^{B3} animals showed diffuse immunoreactivity in wing imaginal discs (Fig. 2B).

Another important feature observed from *psn*^{B3} mutant wing imaginal discs were severely impaired lateral inhibition within sensory organ precursor (SOP) cells, resulting in enlarged SOP territories.⁽⁹⁾ We used rat MAB-Elav antibodies to show the neural hyperplasia in wing imaginal discs in *psn*^{B3} mutant animals (Fig. 2F). Strongly stained clusters of supernumerary SOP cells were revealed. In contrast, there were no rat-MAB-Elav signals in wing imaginal discs in *W1118* (Fig. 2G) or *PEPC-Psn* + 14 WT; *psn*^{B3} line 8 (Fig. 2I). However, a few SOP cells labeled by MAB-Elav were observed from wing imaginal discs in *PEPC-Psn* + 14 WT; *psn*^{B3} line 1 (Fig. 2H). This result suggested that transgenic expression of *Psn* + 14 WT proteins was enough to restore severely impaired lateral inhibition within SOP cells in *psn*^{B3} mutant animals.

In this study, we generated and characterized MAB Psn2G6 recognizing endogenous and transgenically expressed *Psn* proteins *in vivo*. By utilizing four different *psn* alleles and two independent transgenic lines, the specificity of MAB Psn2G6 was confirmed. Thus, MAB Psn2G6 will allow us to examine the precise expression patterns of *Psn* and their roles in development and FAD pathogenesis in *Drosophila*.

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Address reprint requests to:
 Young Ho Koh
 Ilsong Institute of Life Science
 Hallym University
 1605-4 Kwanyang-dong
 Dongan-gu, Anyang
 Gyeonggi-do, 431-060
 Republic of Korea

E-mail: kohyh@hallym.ac.kr

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