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Clearance of Extracellular and Cell-Associated Amyloid β Peptide through Viral Expression of Neprilysin in Primary Neurons[1]

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Amyloid β peptide (Aβ), the pathogenic agent of Alzheimer's disease (AD), is a physiological metabolite constantly anabolized and catabolized in the brain. We previously demonstrated that neprilysin is the major Aβ-degrading enzyme *in vivo*. To investigate whether or not manipulation of neprilysin activity in the brain would be an effective strategy for regulating Aβ levels, we expressed neprilysin in primary cortical neurons using a Sindbis viral vector and examined the effect on Aβ metabolism. The corresponding recombinant protein, expressed in the cell bodies and processes, exhibited thiorphan-sensitive endopeptidase activity, whereas a mutant neprilysin with an amino acid substitution in the active site did not show any such activity. Expression of the wild-type neprilysin, but not the mutant, led to significant decreases in both the Aβ40 and 42 levels in the culture media in a dose-dependent manner. Moreover, neprilysin expression also resulted in reducing cell-associated Aβ, which could be more neurotoxic than extracellular Aβ. These results indicate that the manipulation of neprilysin activity in neurons, the major source of Aβ in the brain, would be a relevant strategy for controlling the Aβ levels and thus the Aβ-associated pathology in brain tissues.

Key words: Alzheimer's disease, amyloid β peptide, neprilysin, neutral endopeptidase, Sindbis virus.

Amyloid β peptide (Aβ), generated through sequential limited proteolysis of amyloid precursor protein (APP), plays a central role in the pathogenesis of Alzheimer's disease (AD) by accumulating in the brain in an aging-dependent manner (*1, 2*). Reduction of the Aβ burden in the brain is thus one of the fundamental strategies for prevention of and therapy for AD. Importantly, Aβ is a physiological metabolite, the steady-state level of which is strictly regulated by the balance between the anabolic and catabolic activities (*3*). Because only a 1.5-fold increase in a particular form of Aβ leads to the aggressive presenile pathology in early-onset familial AD and Down's syndrome cases (*4*), even subtle alterations in this metabolic balance are likely to influence the way Aβ accumulates in the brain with aging.

Therefore, one strategy for reducing the Aβ burden is to up-regulate the catabolic activity involving Aβ-degrading peptidase(s). Although a number of peptidases have been shown to be capable of proteolyzing Aβ in test tube and cell culture paradigms (*5–20*), the only peptidase proven by reverse genetics to participate in Aβ metabolism *in vivo* is neprilysin (*21–23*). Neprilysin is a type II membrane-associated metalloendopeptidase, the catalytic site of which resides on the extracytoplasmic side (*24*), where Aβ is generated and released. Moreover, neprilysin is the most potent Aβ-degrading enzyme among the M13 endopeptidase family members sensitive to neutral endopeptidase inhibitors such as phosphoramidon and thiorphan (*25*). This peptidase thus is presently the most pertinent candidate as a tool for clearing Aβ from the brain enzymatically.

In the present study, we aim to address whether or not the expression of neprilysin in primary neurons leads to successful clearance of Aβ *in vitro* and whether or not neprilysin-dependent degradation takes place intracellularly or extracellularly. For this purpose, we chose to use a Sindbis viral vector to express neprilysin because this virus is known to efficiently and selectively infect neurons (*26, 27*). Figure 1A shows the expression of recombinant neprilysin in primary neurons observed on Western blot analysis. Together with wild-type neprilysin (w), we also expressed an inactive mutant (m) as a negative control. Both proteins appeared in equivalent amounts with apparent molecular weights of 100 kDa within 8 h after infection. The amounts increased in viral dose– and time-dependent manners. In accordance, the thiorphan-sensitive neutral endopeptidase activity hydrolyzing carbobenzoxy-alanyl-alanyl-leucine-*p*-nitroanilide (Z-AAL-*p*NA) appeared in the neurons expressing the wild-type neprilysin in a similar manner (Fig. 1B). The mutant showed no enzymatic activity, as previously reported (*28*), or no apparent dominant negative effect on the endogenous neutral endopeptidase activity. The endoge-

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Abbreviations: Aβ, amyloid β peptide; APP, amyloid precursor protein; AD, Alzheimer's disease; Z-AAL-*p*NA, carbobenzoxy-alanyl-alanyl-leucine-*p*-nitroanilide; MAP-2, microtubule-associated protein-2; GFAP, glial fibrillary acidic protein; HBSS, Hank's balanced salt solutions; ELISA, enzyme-linked immunosorbent assay.

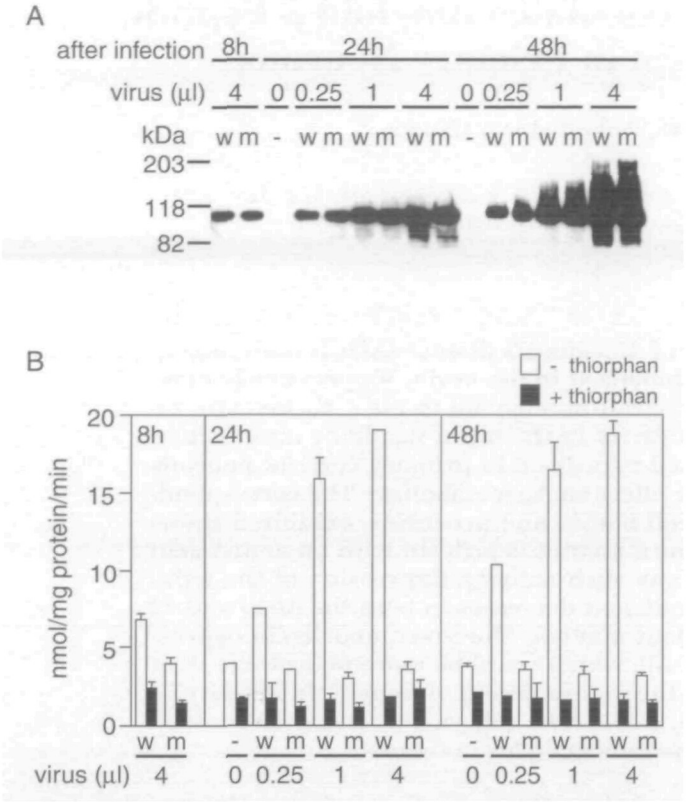

Fig. 1. **Expression of neprilysin in primary cortical neurons with a Sindbis viral vector.** Primary cortical neurons cultured in 3.57 cm wells in a 6-well plate were infected with 0, 0.25, 1, and 4 μl of viral solutions to express the wild-type (w) and mutant (m) neprilysin proteins, and then incubated for 8, 24, and 48 h, as indicated. A: Western blot analysis with anti-neprilysin monodonal antibodies. The molecular weight markers employed were 203, 118, and 82 kDa. B: Neutral endopeptidase activities of cell lysates in the absence (open columns) and presence of thiorphan (closed columns). Each column represents the average of two measurements and the deviation.

nous activity may be attributed to other neprilysin family members (*25*) or proteasome (*29*) because neprilysin was not detectable in uninfected cells on Western blot analysis. Excessive expression seems to cause degradation and aggregation of neprilysin, regardless of the enzymatic activity, as indicated by the faster and slower migrating bands, respectively, on Western blot analysis (see lanes 8, 9, 15, and 16 in Fig. 1A). The expression of endogenous APP remained unchanged in the infected cells (data not shown).

We then confirmed the neuronal expression of recombinant neprilysin by means of immunofluorescence using an antibody to microtubule-associated protein-2 (MAP-2) as a marker for neurons (Fig. 2). Subsets of MAP-2–immunoreactive neurons expressed neprilysin in the cell bodies and processes. The efficiency of infection, as defined as the ratio of neprilysin-positive neurons to MAP-2–positive ones, was 12.9 and 14.1% for the wild-type and mutant neprilysin, respectively, when 4 μl of the viral solution was used under the present experimental conditions. Viral infection did not cause any notable morphological changes of the cells.

These observations indicated that we successfully expressed enzymatically active and inactive neprilysin proteins in equivalent amounts in neurons. We thus investigated the effect of neprilysin expression on the metabolism of Aβ generated endogenously by the primary neurons. The primary neurons secreted sufficiently measurable amounts of Aβ40 and Aβ42, Aβ ending at residues 40 and 42, respectively. Aβ42, although generated physiologically in smaller amounts than Aβ40, is considered to be primarily more pathogenic (*2*). Figure 3 shows the extracellular Aβ levels in the culture media of neurons with and without viral infection. Clearly, the expression of the wild-type neprilysin, but not that of the enzymatically inactive mutant, resulted in reduction of both Aβ40 and Aβ42 in a viral dose–dependent manner at 24 h after the infection. It is notable that the decreases in the Aβ levels correlated well with the enzymatic activities. The ability of active neprilysin to metabolize Aβ lasted for at least 48 h. The results indicate that the expression of neprilysin in neurons leads to effective clearance of extracellular Aβ. Although it is possible that some portion of Aβ was degraded intracellularly prior to secretion (see below), the relatively low efficiency of viral infection indicates that the majority of Aβ degradation took place in a post-secretory manner. The relative decreases in Aβ40 and Aβ42 at 24 h were 29.7 and 35.5% with 0.25 μl of virus, 64.4 and 57.4% with 1 μl, and 73.8 and 66.7% with 4 μl, respectively, indicating that both Aβ species were similarly degraded by neprilysin despite the lower concentration of Aβ42 than that of Aβ40 as a substrate in the culture media. It is notable that the Aβ concentrations in the media are $>10^3$-fold lower than the enzymologically determined K_m values of neprilysin for Aβ (*22, 25*). We thus speculate that neprilysin may be capable of degrading Aβ even more efficiently *in vivo* than *in vitro* if the enzyme and substrates are colocalized in a concentrated manner.

Because the active site of neprilysin is located on the extracytoplasmic side of cell membranes, where Aβ is generated from APP, it is possible that neprilysin may also degrade Aβ intracellularly prior to secretion. To address this question, we examined the effect of neprilysin expression on the cell-associated Aβ. For this purpose, it was necessary to culture cortical neurons on a larger scale, *i.e.* 10 cm dish, because the amounts of cell-associated Aβ were much smaller than those in the media. The efficiency of infection, as determined by neprilysin/MAP-2 immunofluorescence observation, was 23.3 and 21.5% for the wild-type and mutant neprilysin, respectively, in this case. As shown in Fig. 4, both Aβ40 and Aβ42, extractable with guanidine hydrochloride from the cells, were significantly reduced by the wild-type neprilysin, but not by the mutant. The data support the degradation of cell-associated Aβ by the recombinant neprilysin. However, it is also possible that the post-secretory degradation may have partly contributed to the decreases in the cell-associated Aβ by altering the dynamic equilibrium between the extracellular and cell-associated Aβ. Accordingly, the relative decreases in Aβ40 and Aβ42 associated with the cells were 58.0 and 42.7%, respectively, whereas those in the media were higher, *i.e.* 96.6 and 84.4%, respectively. Although we can not determine the relative contributions of intracellular and extracellular processes to the decreases in cell-associated Aβ, a plausible interpretation would be that the extracellular processes account for the portion exceeding the efficiency of viral infection. In any case, the degradation of cell-associated Aβ by neprilysin is consistent with the observations that both

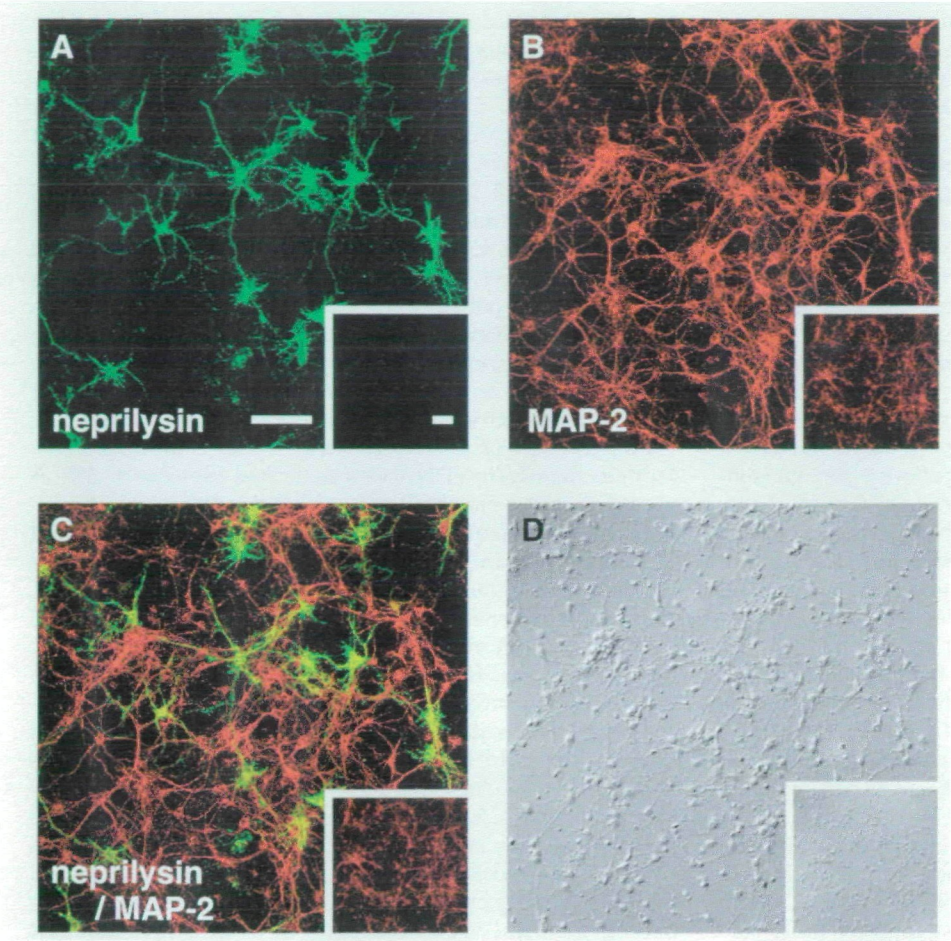

Fig. 2. **Immunofluorescence observation of neprilysin and MAP-2 in cultured neurons.** Primary cortical neurons cultured in 3.57 cm wells were infected with 4 μl of the viral solution for the wild-type neprilysin for 24 h, and then subjected to immunofluorescence analysis with antibodies to neprilysin and MAP-2 as described under "MATERIALS AND METHODS." A and B: Green and red fluorescence signals corresponding to neprilysin and MAP-2, respectively. C: Superimposed image of panels A and B. Yellow indicates colocalization of the neprilysin and MAP-2 immunoreactivities. D: Differential interference-contrast microscopy. Insets show uninfected neurons subjected to immunofluorescence analysis in an identical manner. Scar bar, 100 μm

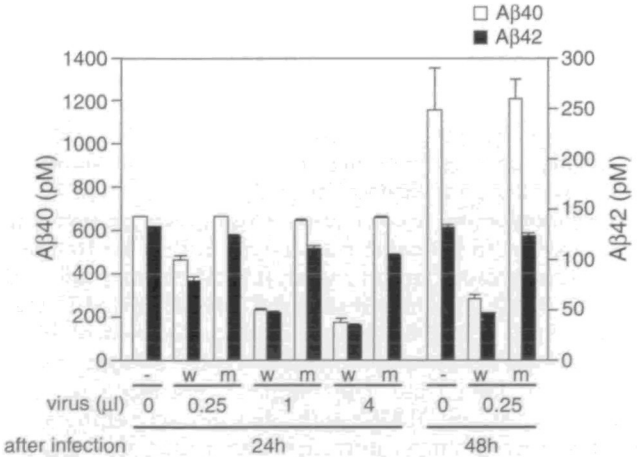

Fig. 3. **Effect of neprilysin expression on the extracellular Aβ40 and Aβ42 levels.** Cultured neurons were infected with viral vectors for the wild-type (w) and mutant (m) neprilysins as described in Figs. 1 and 2. The Aβ40 and Aβ42 levels in the culture media at 24 h after infection with varying volumes of the viral solution and at 48 h after infection with 0.25 μl of the viral solution were determined. Open and closed columns indicate Aβ40 and Aβ42, respectively. The maximum levels of Aβ40 and Aβ42 were 1,400 and 300 pM, respectively. Each column represents the average of two measurements and the deviation.

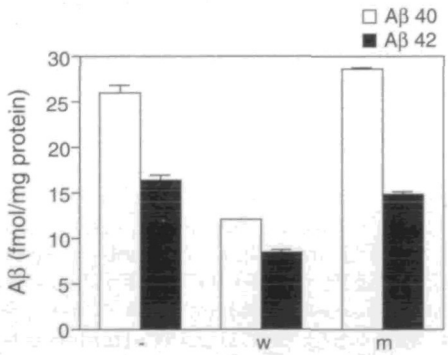

Fig. 4. **Effect of neprilysin expression on the cell-associated Aβ40 and Aβ42 levels.** Primary neurons cultured in 10 cm dishes were infected with 40 μl of the viral solutions for 48 h, and then subjected to quantification of cell-associated Aβ as described under "MATERIALS AND METHODS." Open and closed columns indicate Aβ40 and Aβ42, respectively. Each column represents the average of two measurements and the deviation. The results were confirmed by at least two independent experiments. w: wild-type neprilysin; m: mutant.

APP and neprilysin are axonally transported to termini in neurons (*30–32*), and that they are also associated with the detergent-insoluble glycolipid-enriched membrane domain (*33–35*).

In the present study, we demonstrated that viral expression of neprilysin in cultured neurons leads to effective

clearance of Aβ *in vitro*. This observation provides the first experimental basis for the use of neprilysin activity to reduce Aβ levels, agreeing with our previous speculation based on the difference in the amounts of Aβ between the neprilysin$^{+/+}$ and neprilysin$^{+/-}$ mice (*23*). Moreover, the results raise the possibility of the application of gene therapy to the treatment of AD, although other approaches such as transcriptional up-regulation of endogenous neprilysin expression are also possible.

The advantages of the use of neprilysin activity to dispose of Aβ are as follows. [1] Because neprilysin is a constitutively active peptidase expressed in neurons, modest neprilysin up-regulation would not cause any acute-phase disturbances. [2] Because neprilysin degrades peptides smaller than 4–5 kDa (*24*) without any proteolysis of structural proteins, the effect would not be destructive. [3] Because neprilysin exhibits similar affinity for its substrates including Aβ (*22, 36*), substrates present in abnormally large amounts, *i.e.* Aβ in AD brains, would be selectively degraded. Note that the Aβ42 levels in AD brains are 10^3–10^4-fold higher than those in normal brains without Aβ plaques (*37, 38*). [4] Neprilysin can degrade unphysiologically large amounts of Aβ in the brain, as demonstrated by *in vivo* injection experiments (*21*). Although we do not expect that neprilysin degrades polymerized Aβ, reduction of monomer Aβ will result in reduction of polymer Aβ because these different forms of Aβ exist in a dynamic equilibrium (*39–41*), as demonstrated in the case of polyglutaminyl peptide aggregation *in vivo* (*42*).

These advantages render the neprilysin-based strategy complementary to the other Aβ-lowering strategies, including those employing secretase inhibitors (*43*) and Aβ vaccination (*44*), both of which could have unpredictable side effects upon long-term administration. Possibly, an appropriate combination of these different approaches may eventually lead to the maximum clearance of Aβ from the brain in a manner analogous to the success of the "cocktail therapy" for the Acquired Immunodeficiency Syndrome (*45*).

MATERIALS AND METHODS

Antibodies—Mouse monoclonal antibodies to the extracellular domain of human neprilysin, 56C6, mouse monoclonal anti–MAP-2 antibodies, and rabbit polyclonal anti–glial fibrillary acidic protein (GFAP) antibodies were purchased from Novocastra Laboratories (Tyne, UK), Sigma (St. Louis, MO, USA), and Dako (Carpinteria, CA, USA), respectively. Rabbit anti–rat MAP-2 antiserum and rabbit anti–APP antiserum C4 were originally raised against a rat MAP-2 protein purified from rat brain (*46*) and a synthetic peptide of APP 666-695 according to the numbering of APP695 (*35*), respectively.

Primary Culture of Mouse Cortical Neurons—Cortical neurons were prepared from embryonic C57BL/6CrSlc mice at the developmental stage of 16 to 18 days. The embryos were removed from the uterus and then placed in HBSS (Life Technologies, Grand Island, NY, USA). Cerebral cortices were dissected from the brains of the embryos, freed from meninges, minced, dissociated in 0.25% trypsin (Difco, Sparks, MD, USA) and 0.05% DNase I (Roche Diagnostics, Mannheim, Germany), and then triturated by pipetting. The cells were washed with HBSS and then resuspended in Neurobasal medium (Life Technologies) with 2% B27 (Life Technologies), 0.5% Insulin-Transferrin-Selenium-A supplement (Life Technologies), 0.5 mM glutamine (Life Technologies), and penicillin/streptomycin (Life Technologies), followed by filtration through a Cell Strainer (Falcon, UK). Cortical neurons were plated at a density of 1×10^6 cells/3.57 cm well on a 6-well plate (Falcon) or 1×10^7 cells/10 cm dish (Falcon) coated with 100 μg/ml poly-L-lysine (Sigma), and then kept at 37°C under humidified 95% air and 5% CO_2. The volumes of medium were 2 and 10 ml, respectively. For immunofluorescence analysis, cells were plated on poly-L-lysine coated 13 mm coverslips. The cellular composition of the culture was evaluated by immunofluorescence analysis using mouse anti–MAP-2 and rabbit anti-GFAP antibodies as neuronal and glial-specific markers, respectively, <5% glia being found.

Construction of a Sindbis Viral Vector and Infection—Human neprilysin cDNA was described previously (*25*). A mutation in the zinc-binding motif of neprilysin, which renders the peptidase inactive through amino acid substitution of Glu at 585 to Val (*28*), was introduced using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Both the wild-type (w) and mutant (m) cDNAs were ligated into a pSinRep5 vector (Invitrogen, San Diego, CA, USA). The recombinant RNAs were transcribed from the *Not*I-linearized DNAs with an InvitroScript CAP SP6 *in vitro* transcription kit (Invitrogen) and then cotransfected with DH (26S) helper RNA into baby hamster kidney cells using the Gene Pulser II electroporation system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The supernatants containing the pseudvirions were collected at 48 h after transfection and stored frozen at –80°C in aliquots until use. Infection was carried out by adding the viral vector solutions to cortical neurons that had been cultured for 7–12 days.

Western Blot Analysis and Peptidase Assay—Cortical neurons were lysed in 1% Triton lysis buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, a protease inhibitor cocktail, Complete EDTA-free (Roche), at the concentration suggested by the manufacturer, 1 μM pepstatin A (Peptide Institute, Osaka), and 1% Triton-X100] for 1 h at 4°C and then centrifuged at 20,000 ×*g* for 10 min. Aliquots of the supernatants were subjected to Western blot analysis and a neutral endopeptidase assay with Z-AAL-*p*NA (Peptide Institute) in the presence and absence of thiorphan (Sigma), as previously described (*25*).

Immunofluorescence Analysis—Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, autoclaved in 0.01 M citrate buffer (pH 6.0) for 5 min for antigen retrieval, permeabilized in 0.2% Triton-X100 in PBS for 2 min, and then blocked with blocking buffer (PBS containing 5% skim milk, 5% goat serum, and 0.05% Tween 20). The samples were incubated with 56C6 and anti–rat MAP-2 antibodies in the blocking buffer for 1 h at room temperature, and then with Alexa 488–conjugated goat anti–mouse IgG and Alexa 586–conjugated goat anti–rabbit IgG (Molecular Probes, Eugene, OR, USA) for 1 h at 4°C. The coverslips were washed with PBS and then with distilled water, and then mounted in PermaFluor Aqueous mountant (Immunon, UK). The fluorescence signals were visualized under a FLUOVIEW FV300 confocal microscope (Olympus, Tokyo).

Quantification of Aβ—We quantified Aβ40 and Aβ42 by means of enzyme-linked immunosorbent assays (ELISA)

using sets of monoclonal antibodies, BNT77/BA27 and BNT77/BC05, respectively, as previously described (*47, 48*). Culture media of primary neurons were collected at the indicated after infection times and then subjected to ELISA to quantify extracellular Aβ. The cell-associated Aβ was extracted from extensively washed cells by freezing and thawing in 50 mM Tris-HCl (pH 7.5) containing 6 M guanidine-HCl and EDTA-free Complete three times, incubated for 30 min at room temperature, and then centrifuged for 30 min at 204,000 ×*g* at 20°C. Twentyfold diluted supernatants containing 200 μg protein were subjected to ELISA.

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