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Membrane Localization of β-Amyloid 1–42 in Lysosomes

A POSSIBLE MECHANISM FOR LYSOSOME LABILIZATION[*] [S]

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β-Amyloid peptide (Aβ42) is the core protein of amyloid plaque in Alzheimer disease. The intracellular accumulation of Aβ42 in the endosomal/lysosomal system has been under investigation for many years, but the direct link between Aβ42 accumulation and dysfunction of the endosomal/lysosomal system is still largely unknown. Here, we found that both *in vitro* and *in vivo*, a major portion of Aβ42 was tightly inserted into and a small portion peripherally associated with the lysosomal membrane, whereas its soluble portion was minimal. We also found that the Aβ42 molecules inserted into the membrane tended to form multiple oligomeric aggregates, whereas Aβ40 peptides formed only dimers. Neutralizing lysosomal pH in differentiated PC12 cells decreased the lysosomal membrane insertion of Aβ42 and moderated Aβ42-induced lysosomal labilization and cytotoxicity. Our findings, thus, suggest that the membrane-inserted portion of Aβ42 accumulated in lysosomes may destabilize the lysosomal membrane and induce neurotoxicity.

Alzheimer disease (AD)[3] is the most common age-related neurodegenerative disorder, and the production and cerebral deposition of β-amyloid peptide (Aβ) is widely believed to be central to the development of AD (1, 2). The main isoforms of Aβ in AD are Aβ40 and Aβ42 (containing 40 and 42 amino acids, respectively) generated from the proteolytic processing of amyloid precursor protein (APP) (3). Aβ42 is more neurotoxic than Aβ40 and is the principle species associated with amyloid plaque (4–7), but the exact molecular mechanisms of how Aβ42 damages the neurons and deposits in brain still remain unclear.

Classically, extracellular deposition of Aβ was thought to be important in AD pathogenesis. More recent evidences have demonstrated that intraneuronal Aβ may play a crucial role in the early progression of the disease and pointed toward the importance of endosomal/lysosomal compartments in this pathogenic process (8–12). The endosome/lysosome pathway participates in Aβ production (13–18), and Aβ-released outside neurons in soluble or aggregated form can also be re-internalized and act inside endosomal/lysosomal compartments (19). Nixon and co-workers (20) suggested that autophagosomes and other prelysosomal vacuoles are involved in AD. Recently Ling *et al.* (21) reported that Aβ42 expression, but not that of Aβ40, in *Drosophila* induces an age-dependent impairment of neuronal autophagy at a post-lysosomal stage, leading to extensive neuronal damage and death. Yang *et al.* (22, 23) reported that loss of lysosomal membrane integrity occurs in response to Aβ42 accumulation and is an early event in neuron death. Previous studies demonstrated that internalized Aβ42 is largely resistant to degradation and accumulates as insoluble aggregates in late endosomes or secondary lysosomes in a variety of cells (24–27); in contrast, shorter peptides such as Aβ40 are rapidly degraded and do not accumulate (24, 25, 28). Notably, careful studies of human brain and brains from Alzheimer transgenic mice using C-terminal-specific antibodies against Aβ40 and Aβ42 established that most of the intraneuronal Aβ end at residue 42, not at residue 40, and are frequently co-localized with cathepsin D, a lysosomal marker (8, 29). The oligomeric Aβ has been found to be most pathogenic (30–32). In tissue derived from the human brain, Aβ oligomerization initiates within cells rather than in the extracellular space (33). Others have reported that Aβ oligomerization could occur in the endosomal compartments (34, 35). The low pH of endosomes and lysosomes and their ability to concentrate solutes may provide an ideal environment in which to promote amyloid fibril assembly (24, 25). Overall, the interaction between Aβ42 and the lysosomal system seems to be pivotal for the preferential accumulation of Aβ42 in neurons and association with AD pathogenesis.

Here, we show that a major portion of Aβ42 accumulated in lysosomes was inserted into the lysosomal membrane, where they remained undegraded. We also present evidence that the multiorder oligomer of Aβ42 formed in association with the lysosome membrane at low pH. The pH-dependent membrane insertion of Aβ42 could cause membrane instability and lysosomal leakage. Our findings provide a possible mechanism for the lysosomal accumulation of Aβ42 and its association with lysosome disruption, which have been hypothesized to be involved in AD pathology.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S6.

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[3] The abbreviations used are: AD, Alzheimer disease; Aβ, β-amyloid peptide; APP, amyloid precursor protein; BafA1, bafilomycin A1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HB, homogenization buffer; mN, millinewtons; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PC, phosphatidylcholine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

MATERIALS AND METHODS

Aβ Peptides, Antibodies, and Reagents—Lyophilized Aβ (AnaSpec Co.) was dissolved in dimethyl sulfoxide to obtain a 2 mM stock solution that was centrifuged (15,000 × *g*) for 10 min to remove insoluble particulates (36) and stored at −70 °C until use. Monoclonal antibody 6E10, which recognizes an epitope within residues 1–17 of human Aβ, was purchased from Chemicon International Inc. Rabbit polyclonal antibody to the human C terminus of Aβ42, which does not cross-react with Aβ40 or APP, was from Signet Laboratories Inc. Mouse monoclonal antibody to human C terminus of Aβ40, which does not cross-react with Aβ42, was from Upstate Biotechnology. Antibodies against Lamp-1, Rab7, Rab6, and calnexin were from Santa Cruz Biotechnology. Secondary antibodies were obtained from ZhongShan Biotechnology. All lipids, bafilomycin A1 (BafA1), chloroquine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lucifer Yellow, *p*-nitrophenyl phosphate, 4-methylumbelliferyl-β-D-galactosidase, and a protease inhibitor mixture were purchased Sigma. Nerve growth factors and G418 were products of Invitrogen. All the other chemicals used were of analytical grade and were manufactured in China.

Monolayer Surface Pressure Measurements—Monolayer surface pressure (π), defined as the change of surface tension after spreading a monolayer on water surface, was measured with a NIMA 9000 microbalance. Experiments were conducted as described in our previous work (37). Lipids were dissolved in a solvent of chloroform/methanol (7:1 v/v, 1.0 mg/ml). The maximum increase of surface pressure ($\Delta\pi$) induced by self-penetration of Aβ42 into the air-water interface without a lipid monolayer was 15.6 mN/m, and the minimum concentration of Aβ42 to reach such a maximum $\Delta\pi$ was 800 nM, determined by pre-experiments (data not shown). Thus, we kept initial surface pressure (π_i) of the lipid monolayers at or above 16.0 mN/m and the concentration of Aβ42 at 800 nM throughout experiments.

Hydrolysis of Aβ42 and MALDI-TOF MS—Preparation of small unilamellar vesicles and hydrolysis of Aβ42 was performed in accordance with the procedure described in our previous work (37). Lipids of the desired composition were mixed in chloroform/methanol (7:1, v/v) and dried under a stream of nitrogen. Lipid films were resuspended and sonicated in 5 mM Na_2HPO_4-HAc to near optical clarity. The concentration of phospholipid was determined (38). In a typical experiment Aβ42 was incubated in the presence or absence of vesicles at 37 °C. Hydrolysis reaction was initiated by the addition of papain, the specific hydrolytic sites of which are Glu-X, Gly-X, Tyr-X, His-X, Lys-X, and Arg-X. After 30 min, iodoacetamide was injected to stop the reaction. Then the mixture was subjected for MALDI-TOF MS analysis with a BIFLEX III MALDI-TOF mass spectrometer (Bruker Daltonics).

Cell Culture and Differentiation—Rat pheochromocytoma (PC12) cells were maintained in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 mg/ml streptomycin. For neuronal differentiation, PC12 cells were cultured with serum-free RPMI 1640 medium containing 1% bovine serum albumin, and 50 ng/ml 2.5 S nerve growth factor for up to 8 days. Mouse neuroblastoma N2a cells double-transfected with cDNAs encoding human βAPP harboring the "Swedish" mutant (βAPPswe) and PS1 (N2a APPswe×PS1) were maintained in medium containing 45% Dulbecco's modified Eagle's medium, 50% Opti-MEM, 5% fetal bovine serum, antibiotics, and 0.2 mg/ml G418.

Preparation and Characterization of Lysosome—The procedure was processed as described (39). Briefly, cells were harvested by trypsinization and washed with phosphate-buffered saline and homogenization buffer (HB; 0.25 M sucrose, 10 mM Hepes, 1 mM EDTA (pH 7.4)). Hereinafter all manipulations were carried out at 4 °C using pre-cooled reagents. Washed cells were resuspended in HB supplemented with protease inhibitor mixture at 1×10^8/ml and then homogenized using a Dounce glass Teflon homogenizer. Homogenates were spun at 800 × *g* for 10 min to pellet nuclei and unbroken cells, which were then rehomogenized in a half-volume of HB. Supernatant was combined and centrifuged at 3000 × *g* for 10 min to remove large heavy mitochondria. The resultant supernatant subsequently was centrifuged for 10 min at 18,000 × *g*, obtaining a pellet resuspended in HB. The resuspension (0.5 ml) was layered on 6.5 ml of iso-osmotic (0.25 M sucrose) Percoll (GE Healthcare) at a concentration of 30% (pH 7.4), with a 1-ml cushion of 2.5 M sucrose at the bottom. After centrifuging at 44,000 × *g* for 40 min in a fixed-angle rotor (Hitachi P70AT2), fractions of ~0.5 ml were carefully collected from the top of tube. Percoll was removed as described (40). Proteins were measured with BCA protein assay kit (Pierce). Organelle markers assayed for lysosomes were acid phosphatase (39) and β-hexosaminidase (41).

For preparation of a mouse brain lysosomal fraction, equal amounts of brain tissue derived from analogous cortical regions or hippocampus from brains of APP×PS1 transgenic or wild type mice sacrificed at different ages (2 and 10 months) were processed as described for cells with slightly modifications. After dissection, brain tissue was immersed immediately in ice-cold HB and homogenized. Homogenate was digested with DNase I (250 μg/ml for 30 min) and centrifuged at 1000 × *g* for 10 min to remove nuclei and intact cells. The supernatant was collected and centrifuged again to remove blood cells. Next purification of lysosomes was carried out as described above.

Latency Measurements—Intactness of lysosomes was assessed by measuring the activity of β-hexosaminidase under isotonic conditions with or without 0.1% Triton X-100. Latency (%) of lysosomes is expressed as (activity with detergent minus activity without detergent)/(activity with detergent) × 100. When the effect of Aβ on the intactness of lysosome was tested, lysosomes were incubated in the absence or presence of Aβ for 30 min before conducting latency measurement. The buffer used in the experiment was 5 mM citrate/phosphate (pH 4.5 or 7.4, isotonic osmolarity was adjusted with sucrose).

Lysosomal Subfractionation—All the fractionation procedures were conducted at 0–4 °C. Soluble (luminal) lysosomal proteins were obtained by resuspending the lysosomes in phosphate-buffered saline, freeze/fracturing them in dry ice/ethanol, and removing membranes by ultracentrifugation for 30 min at 200,000 × *g*. The membrane pellet was incubated with

0.1 M Na_2CO_3 (pH 11.0) (42) for 30 min and spun as above to give peripheral membrane proteins. The pellet of carbonate-washed membrane was further stripped by incubation with 1 M NaCl (43) for 30 min and spun as above. The ultimate membrane pellet was solubilized in 2% SDS and designated as membrane-inserted proteins. All buffers were supplemented with protease inhibitor mixture.

In Vitro Incubation with Biological Membranes—Samples containing 100 μg of protein of isolated PC12 lysosomes were sonicated on ice for 15 s and ultracentrifuged at 200,000 × *g* for 30 min. The obtained membrane pellet was resuspended in 100 μl of Na_2HPO_4-citric acid buffers (20 mM, pH 4.5 or 7.4). Freshly dissolved Aβ peptides (0.1 μM) were added and incubated at 37 °C for 60 or 120 min. After incubation, the samples were ultracentrifuged as above to yield a supernatant (free unlabeled Aβ) and membrane pellet. The pellet was extracted with 0.1 M Na_2CO_3 (pH 11.0) and 1 M NaCl sequentially for loosely attached Aβ. Finally, the pellet containing membrane integrated Aβ was solubilized in 2% SDS. For enzyme hydrolysis, 1 mg/ml trypsin was added to the 60-min incubation mixture of Aβ42 and membrane at 37 °C for 5 or 15 min just before ultracentrifugation.

Immunoblotting—Western blots for Aβ detection were performed as described (44). Analysis was performed using the public domain NIH ImageJ program (available on the Internet).

Measurement of Lysosomal Membrane Instability—Membrane instability and leakage of PC12 lysosomes were assessed by observing the distribution change of lysosomal fluorescent dye Lucifer Yellow or the leakage of lysosomal enzyme β-hexosaminidase into cytosol. For measuring the distribution of Lucifer Yellow, cells were labeled with Lucifer Yellow (0.1 mg/ml) at 37 °C for 16 h and washed 3 times. Labeled cells with or without BafA1 pretreatment were then incubated with Aβ42 at 37 °C for 6 h and visualized on a Nikon E800 microscope. To assess the leakage of β-hexosaminidase, cells that had been incubated with Aβ42 in the presence or absence of BafA1 were collected and homogenized ten strokes. Cytosol fraction was obtained by ultracentrifugation, and the activity of β-hexosaminidase was measured as described (41). The leakage of lysosomal β-hexosaminidase was expressed as a percentage of total activity obtained from cell homogenates. Assays were repeated in three independent experiments performed in duplicate. The significance of the results was assessed by Student's *t* test.

For lysosomes in brain, samples from the cortex or hippocampus of 2- and 10-month-old transgenic or wild type mice were homogenized and centrifuged. Then the activity of β-hexosaminidase was assayed as for cells. Purified lysosomal fraction were also incubated in 0.15 or 0.25 M sucrose at 37 °C for 5 min, then the suspension was used for the assay of lysosomal integrity by measuring the activity of β-hexosaminidase (45, 46).

Measurement of Cell Viability—Cell viability assays were carried out by MTT dye conversion assay (47) in 96-well cell culture plates. Cell viability was expressed as a percent of absorption of vehicle-treated control. In addition, cell death was evaluated by measuring the amounts of cytoplasmic lactate dehydrogenase released into medium. Lactate dehydrogenase activities were measured using a CytoTox96 nonradioactive assay kit (Promega), according to the manufacturer's instructions. Cytotoxicity was expressed as percentage of released lactate dehydrogenase per total cellular lactate dehydrogenase content. Assays were repeated in three independent experiments, each performed in triplicate.

RESULTS

Aβ42 Can Insert into the Lipid Membrane via Its C Terminus in a pH-dependent Manner—Previous studies implicate that a likely primary target of Aβ is the membrane, as the peptide may alter many of its important physical and biological properties (48–50). Therefore, we first examined the membrane insertion ability of Aβ42 via monolayer experiments. The surface pressure is believed to increase when the protein penetrates into the monolayer, and as reported earlier, it does not change if the protein only interacts with phospholipid head groups (51, 52). Thus, the increase in surface pressure ($\Delta\pi$) of the lipid monolayer post-injection of proteins into subphase can only be interpreted as the result of actual insertion of the proteins into the phospholipid monolayer. $\Delta\pi$ can be obtained as a function of the initial surface pressures (π_i) for each sample. Plotting a series of $\Delta\pi$ *versus* π_i would yield a straight line with a negative slope, which intersects the abscissa at a point defined as the critical insertion pressure (π_c). The value of π_c is used to evaluate the penetration ability of the protein into this lipid monolayer.

Fig. 1*A* shows $\Delta\pi$-π_i plots of Aβ42 for the total brain lipid monolayer under various pH conditions. The values of π_c at pH 7.0, 6.0, 5.0, and 4.0 were 27.9, 30.6, 32.2, and 34.9 mN/m, respectively. Fig. 1*B* shows the $\Delta\pi$-π_i plots of Aβ42 for an eggPC monolayer under different pH values. The values of π_c for eggPC under pH 7.0, 6.0, 5.0, and 4.0 were 27.7, 30.1, 32.0, and 36.1 mN/m, respectively. Fig. 1*C* gives the correlation between π_c and pH, clearly indicating that the membrane insertion ability of Aβ42 increased with decreasing pH. Same experiments were performed with other phospholipid mixtures, such as PC/phosphatidylethanolamine, PC/sphingomyelin, and PC/cerebroside, with similar effects (data not shown). These data suggest that the membrane insertion ability of Aβ42 is critically dependent on pH and not sensitive to lipid composition. The biological membrane pressure had been established to be 31–34 mN/m (53). The packing density of the lipid monolayer with a surface pressure in this range is assumed to be comparable to that of the lipid bilayer (54, 55). Thus, the monolayer results indicated that Aβ42 is able to insert into a physiological bilayer at a pH lower than 5.5.

To further characterize membrane-associated Aβ42, papain was used to hydrolyze Aβ42 after it reacted with lipid vesicles based on the fact that Aβ42 inserted into a lipid membrane would be resistant to digestion. The hydrolysis products were analyzed by MALDI-TOF MS. The MS peaks from a typical enzyme hydrolysis of the Aβ42 solution were identified and summarized in supplemental Table S1. Fig. 2, *A* and *B*, show the MS spectra of the hydrolysis products of Aβ42 in the absence and presence of vesicles at pH 4.0. The two MS spectra exhibit some obvious differences; the peaks of *m*/*z* 2932, 3672, and 4072 disappeared in the presence of vesicles. In comparison, we

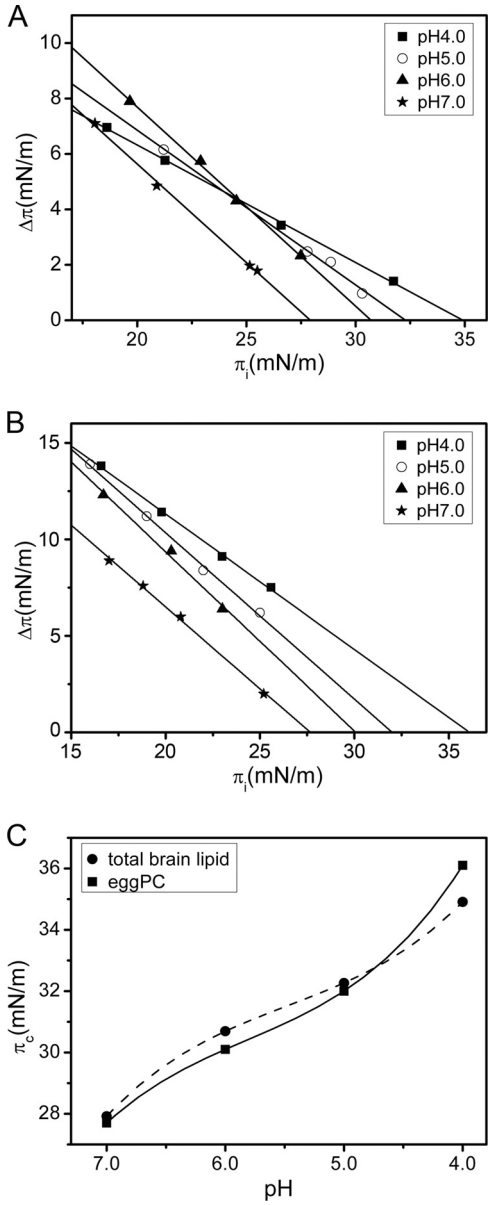

FIGURE 1. **Aβ42 can insert into lipid membranes in a pH-dependent manner.** The interaction between Aβ42 and total brain lipid monolayers (*A*) or eggPC (*B*) was detected via monolayer surface pressure measurements at various pH values. The surface pressure change ($\Delta\pi$) caused by peptide insertion into the lipid monolayer can be obtained as a function of the initial surface pressure π_i. A plot of $\Delta\pi$ *versus* π_i yields a straight line with a negative slope that intersects the *abscissa* at a limiting surface pressure, the critical insertion pressure, π_c. The value of π_c is a quantitative measure to evaluate the insertion ability of the peptide into the lipid monolayer. The concentration of Aβ42 was 800 nM, and Na_2HPO_4-citric acid was the buffer used to supply a sufficient pH range. *Panel C* gives the correlation between the critical insertion pressure π_c and pH.

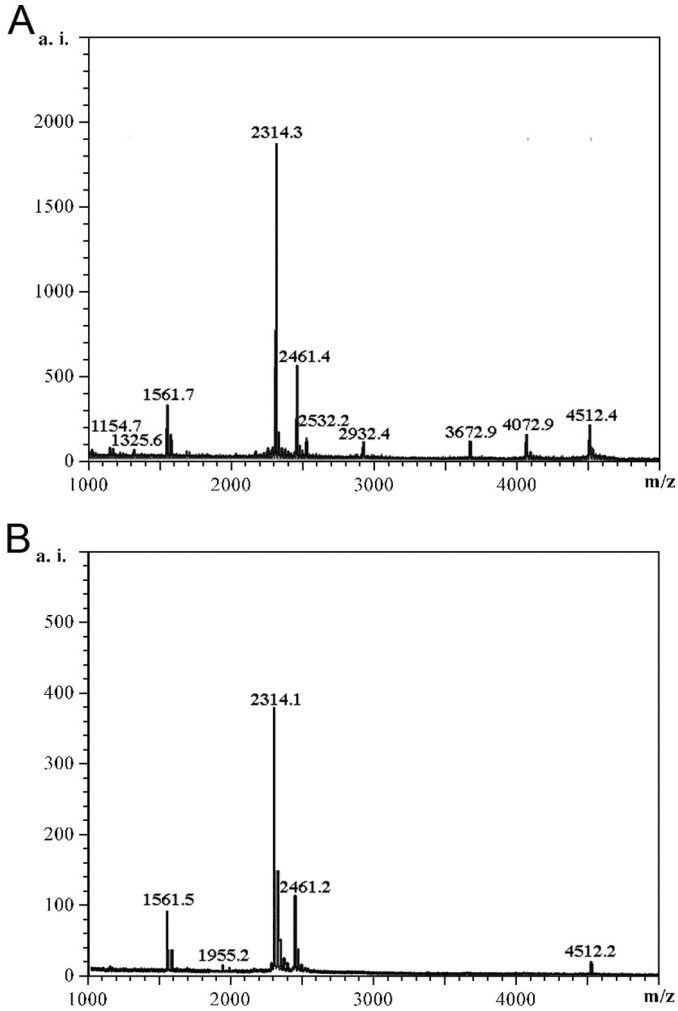

FIGURE 2. **The MS spectra of the hydrolysis products of Aβ42 with or without lipid vesicles at pH 4.0.** MS spectra in the absence (*A*) or presence (*B*) of eggPC were obtained after hydrolysis at 37 °C for 30 min by papain (the peptide/enzyme ratio was 10) in a buffer of Na_2HPO_4-HAc. The hydrolysis fragments are indicated by *m/z*. For each spectrum, only peaks with an *m/z* value higher than 1000 are shown, as other substances in the matrix (such as α-cyano-4-hydroxycinnamic acid and its contaminants) could cause interference at low *m/z*. The identifications of the typical peaks of MS are summarized in supplemental Table S1. *a.i.*, arbitrary intensity.

found that the inaccessible cleavage sites were Gly^{33}-Leu^{34} and Gly^{37}-Gly^{38}, both of which are located in the C-terminal transmembrane domain of Aβ42. In contrast, the mass spectra in the presence and absence of vesicles at pH 7.0 were almost identical (supplemental Fig. S1), indicating that papain could access nearly all the Aβ42 cleavage sites after reacting with vesicles at neutral pH. Therefore, we concluded that Aβ42 inserts into lipid vesicles via its C-terminal at low pH, thus protecting its C-terminal sites from cleavage.

Lysosome-accumulated Aβ42 Mainly Inserts into the Lysosomal Membrane in Cells—To investigate whether Aβ42 could integrate into biological membranes of intracellular acidic organelles, we incubated differentiated PC12 cells with 1 μM freshly dissolved Aβ42 for 6 h at 37 °C, then the cells were washed with phosphate-buffered saline and transferred into fresh medium for 6 h. After incubation, the cells were harvested, and the lysosomes were isolated on a self-generated Percoll gradient as described under "Materials and Methods." The two lysosomal enzyme markers, acid phosphatase and β-hexosaminidase, are shown to be concentrated in fractions 11 and 12, indicating that the bulk of lysosomes was contained in the two fractions (Fig. 3*A*). Intactness of the organelles was above 90% based on a latency measurement. We also characterized our lysosomal preparations by Western blot (supplemental Fig. S2) and electron microscopy (supplemental Fig. S3). The lysosomal proteins Lamp-1 and Rab7 were markedly enriched in the lysosomal fraction, whereas the endoplasmic reticulum

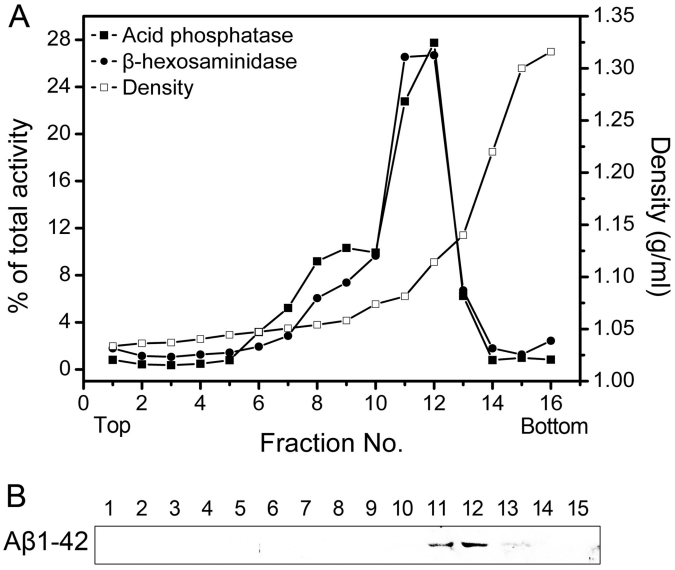

FIGURE 3. **Distribution of accumulated Aβ42 in differentiated PC12 cells.** Differentiated PC12 cells were incubated with 1 μM freshly dissolved Aβ42 for 6 h in binding media and 6 h in fresh media and then homogenized. *A*, postnuclear supernatant was fractionated on a Percoll gradient as described under "Materials and Methods" and analyzed for the distribution of acid phosphatase, β-hexosaminidase, and density. *B*, internalized Aβ42 existed in the fractions with peak values of lysosomal enzyme markers. Gradient fractions were electrophoresed in a 16% Tris-Tricine gel, electroblotted onto a nitrocellulose membrane, and probed with an Aβ42 antibody.

(ER) and Golgi markers calnexin and Rab6 were undetectable, suggesting that the ER and Golgi were not present in this lysosomal fraction. Immunoelectron microscopy analysis further confirmed that the lysosomal fraction was mostly composed of lamp-1-positive lysosomes.

We also examined the subcellular location of endocytosed Aβ42 in PC12 cells. Western blot analysis of gradient fractions with a specific Aβ42 antibody showed that Aβ42 applied extracellularly was enriched in the lysosomal fraction (Fig. 3*B*). Also, immunofluorescence analysis displayed a co-localization of Aβ42 with Lyso Tracker (data not shown). These results indicated that exogenous Aβ42 could be taken up by PC12 cells and located to lysosomes.

To determine the intralysosomal distribution of Aβ42, isolated lysosomes were fractionated into a soluble and a membrane fraction. The membrane fraction was then sequentially extracted with carbonate and sodium chloride, which are widely used to strip peripherally membrane-attached proteins (42, 43). After these treatments, the proteins remaining in the membrane fraction were considered to be tightly inserted into the lysosomal membrane. Thus, the proteins of intact lysosomes were fractionated into "soluble," "membrane-associated" (Na_2CO_3-extractable and NaCl-extractable), and "membrane-inserted" (detergent-extractable) fractions. As controls, β-hexosaminidase, a lysosomal matrix marker, was detected entirely within the soluble fraction (Fig. 4*A*), whereas Lamp-1, a lysosomal membrane marker, was enriched in the membrane-inserted fraction (Fig. 4*B*, *top panel*), indicating that the lysosome was substantially fractionated.

The lysosomal distribution of internalized Aβ42 was shown in Fig. 4*B*, *bottom panel*. The internalized Aβ42 was markedly enriched in the membrane-inserted fraction, whereas only a small portion was extracted by 0.1 M Na_2CO_3, and its soluble portion was nearly undetectable.

Furthermore, we examined the lysosomal localization of the endogenous Aβ42 accumulated in N2a APPswe×PS1-transfected cells. Immunoblots with a specific Aβ42 antibody, Aβ40 antibody, and 6E10 (recognizing residues 1–17 of Aβ) showed that Aβ42 was markedly localized in the membrane-inserted fraction with only a small portion loosely attached to the membrane (Na_2CO_3 extraction), and its soluble portion was also undetectable (Fig. 4*C*, *top panel*). Aβ40, however, was undetected in any fraction of the lysosomes (Fig. 4*C*, *middle panel*), indicating that Aβ42 preferably accumulated in the lysosomes of cells. Combining the results from PC12 and N2a cells, one may suppose that Aβ42 mostly tightly inserted into the lysosomal membrane may play an important role in neuronal toxicity. Additionally, we investigated the subcellular localization of Aβ42 in brains from APP×PS1 transgenic mice using immunoelectron microscopy. The results in supplemental Fig. S4, in agreement with the recent findings (29, 56), clearly showed that the immunoreactivity of intraneuronal Aβ42 increased with aging (there were more gold particles in the lysosomes of a 10-month-old mouse than a young mouse) and localized predominantly to lysosomes of neurons in transgenic mice (comparatively less gold particles in the endoplasmic reticulum and mitochondria).

Effect of Alkalizing Drugs on the Insertion of Aβ42 into the Lysosomal Membrane—To assess the effect of pH on the lysosomal distribution of Aβ42, the lysosomal pH was altered by BafA1 or chloroquine. BafA1 specifically inhibited the transmembrane component of ATPases, the H^+ pump responsible for the acidification of late endosomes and lysosomes (57). Chloroquine, a weak base, accumulated in lysosomes where it raises the pH (58). Differentiated PC12 cells were incubated with 1 μM Aβ42 for 6 h in the presence or absence of 10 nM BafA1 or 10 μM chloroquine, then washed with phosphate-buffered saline and transferred into fresh medium for 6, 12, or 24 h (Fig. 4, *D* and *E*). An equal quantity of isolated lysosomes was fractionated as described above and analyzed by Western blotting. BafA1 induced an obvious effect on the lysosomal distribution of Aβ42, decreasing the level of membrane-inserted Aβ42 from ~90% to nearly 50% (Fig. 4*D*). A similar effect was also observed with chloroquine (Fig. 4*E*), indicating that Aβ42 can insert into the lysosomal membrane in a pH-dependent manner.

Extending the incubation time from 6 to 12 and 24 h, only Aβ42, which tightly integrated into the membrane, remained detectable in the lysosomes, whereas the fraction peripherally attached to the membrane became undetectable (Fig. 4, *E* and *F*). The lysosomal system is known to process or digest cargoes from phagocytosis, endocytosis, and autophagy (59, 60). Therefore, the fact that the membrane-inserted Aβ42 remained undegraded suggests that this form may resist hydrolysis by lysosomal proteases.

Aβ42 and Aβ40 Differ in their Interaction with the Lysosomal Membrane—To explore whether another pathologically important peptide, Aβ40, and a shorter peptide, Aβ28, also inserted into and accumulated at the lysosomal membrane after internalization by differentiated PC12 cells, we incubated syn-

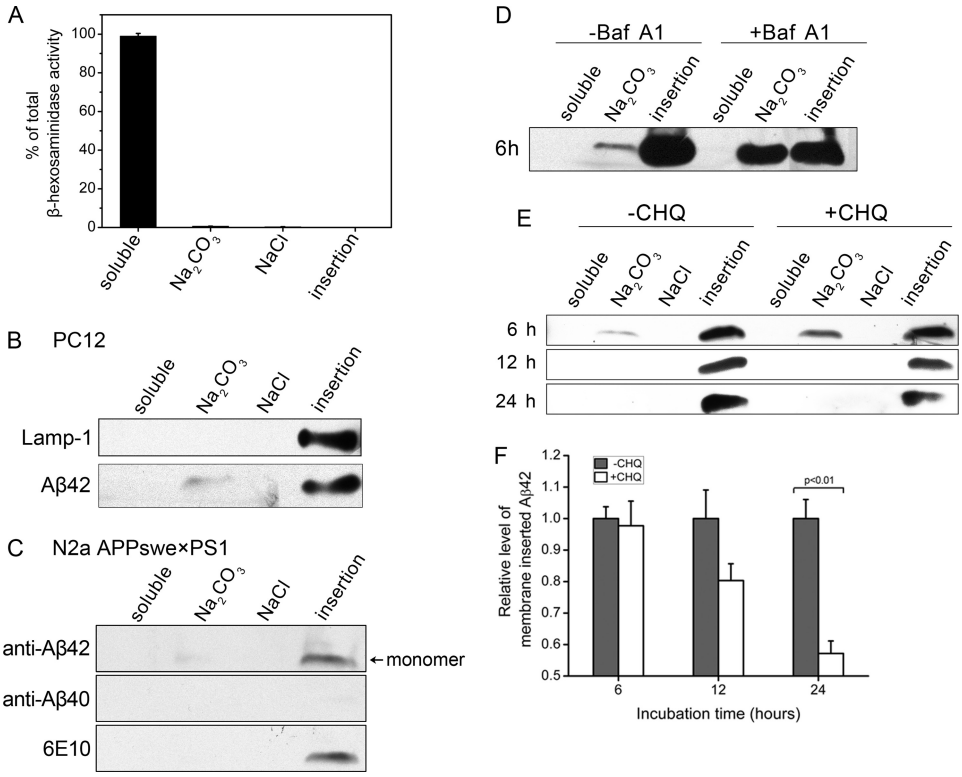

FIGURE 4. **Lysosome accumulated Aβ42 is enriched in the membrane fraction.** Intralysosomal distribution of β-hexosaminidase activity (lysosomal matrix marker (*A*)), Lamp-1 (lysosomal integral membrane protein marker) and internalized Aβ42 in differentiated PC12 cells (*B*) is shown. Purified lysosomes were fractionated into a soluble fraction (soluble), 0.1 M carbonate extracted fraction (Na_2CO_3), 1 M NaCl extracted fraction (NaCl), and membrane-inserted protein fraction (insertion) as described under "Materials and Methods." *C*, the distributions of endogenous Aβ accumulated in lysosomes of N2a APPswe×PS1 cells were examined in each lysosomal fraction with specific anti-Aβ42, anti-Aβ40, and 6E10 antibodies. BafA1 (*D*) or chloroquine (*CHQ*, *E*) reduced the level of the membrane-inserted Aβ42. Differentiated PC12 cells were pretreated with or without alkalizing drugs, BafA1 (10 nM), or chloroquine (10 μM) for 30 min, incubated with 1 μM Aβ42 at 37 °C for 6 h, and then transferred into fresh medium for the indicated times. Lysosomes were purified, and equal volumes were subfractionated and analyzed by Western blotting with an antibody specific to the C terminus of Aβ42, which does not recognize Aβ40 or APP. *F*, a graphic presentation of E shows the membrane-inserted Aβ42 level in the presence or absence of chloroquine. The Aβ42 level in the absence of chloroquine was set at 1, and the data represent those from two individual experiments.

thetic Aβ40 and Aβ28 with cells as Aβ42. Fig. 5 shows the intralysosomal distribution of Aβ42, Aβ40, and Aβ28 after 6 h of incubation. Interestingly, the total amount of the lysosomal accumulation of Aβ42 (that is, the sum of soluble, membrane-attached, and membrane-inserted Aβ42) was remarkably higher than that of Aβ40 and Aβ28. Compared with Aβ42, Aβ40 had only a very low remnant level located at the membrane fraction. Aβ28 appeared to be eliminated to the same background level as the control. Alkalizing drug treatment (*i.e.* cells were preincubated with 10 μM chloroquine for 30 min before Aβ was added) also caused the same effect with Aβ40 as it did with Aβ42; however, the level of Aβ40 in each fraction was very low.

To further clarify the molecular basis of pH affecting the interaction of Aβ peptides with biological membranes, we incubated Aβ monomers of different lengths with purified lysosomal membrane for 60 min in acidic (pH 4.5) or neutral (pH 7.4) buffer *in vitro*. Fig. 6*A* shows the membrane interaction of Aβ monomers, indicating that pH has a significant effect on the distribution of Aβ42 and Aβ40. The levels of membrane insertion and membrane association decreased at pH 7.4, whereas the water-soluble fractions increased compared with those at pH 4.5. In addition, the amount of membrane insertion of Aβ42 was greatly higher than that of Aβ40 at both pH 4.5 and 7.4. Compared with Aβ42, Aβ40 existed mainly in the water-soluble (pH 7.4) or membrane-associated state (pH 4.5). Moreover, regardless of pH 4.5 or 7.4, Aβ28 only existed in the free form without any membrane association (data not shown). Extending the incubation time from 60 to 120 min (Fig. 6*B*) made it clearer that pH plays an important role in Aβ-membrane interactions. Note that at lower pH, Aβ42 preferred to form many multiple-molecular weight oligomeric aggregates in both the membrane-associated and membrane-inserted fractions, whereas Aβ40 occurred only in dimers.

To demonstrate that membrane insertion may protect Aβ42 from hydrolysis by proteases, we treated an incubation mixture of Aβ42 and the lysosomal membrane with trypsin for different times. As expected, trypsin degraded most free and membrane-attached Aβ42 within 15 min, whereas membrane-inserted Aβ42 mostly persisted (Fig. 6*C*). As a control, mixture of Aβ42 with only buffer showed complete degradation within 15 min (data not shown). These data provided further evidence that Aβ42 molecules resist degradation in the membrane-inserted state. Here, we also examined the membrane-insertion characteristics of Aβ42 with an electron microscope. An incubation mixture of Aβ42 and the lysosomal membrane was washed first with carbonate and NaCl to remove loosely attached Aβ42 and then immunogold-labeled with 6E10 (the N terminus antibody of Aβ42) or the C terminus antibody of Aβ42. As expected, the sample stained positively with 6E10 but negatively with the C terminus Aβ42 antibody, further indicating that Aβ42 inserted into the lysosomal membrane through its C terminus (supplemental Fig. S5).

Aβ42 Induces Lysosomal Labilization of PC12 Cells in a pH-dependent Manner—To assess whether pH has an effect on Aβ42-induced lysosomal labilization, we performed a combination of a fluorescence assay and an enzyme activity analysis. For the fluorescence assay, we used Lucifer Yellow, a membrane-impermeable marker of fluid-phase pinocytosis that accumulates in secondary lysosomes, to determine lysosomal leakage as previously described (22, 25). Differentiated PC12 cells were incubated first with Lucifer Yellow and then with Aβ42 for 6 h at 37 °C and examined under a fluorescence microscope. As shown in Fig. 7*A*, untreated control cells dis-

played a punctate pattern of fluorescence, revealing small, circumscribed, vesicular structures resembling intact lysosomes. After treatment with Aβ42, the cells displayed a diffuse intracellular pattern of fluorescence, indicating lysosomal leakage into the cytosol. However, this redistribution of Lucifer Yellow could be counteracted partly if the cells were pretreated with 5 nM BafA1 for 20 min before the addition of Aβ42.

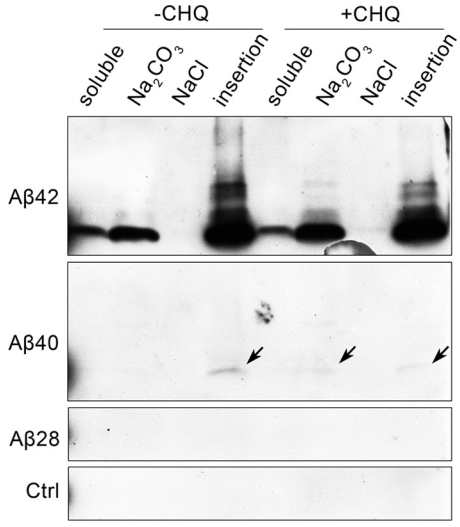

FIGURE 5. **Preferential accumulation of Aβ42 *versus* Aβ40 and Aβ28 in differentiated PC12 cells.** Cells were pretreated with or without 10 μM chloroquine (*CHQ*) for 30 min and then incubated with or without 2 μM Aβ isoforms at 37 °C for 6 h. Lysosomes were purified, and equal amounts of lysosomes were subfractionated into soluble, 0.1 M carbonate-extractable (Na_2CO_3), and 1 M NaCl-extractable (NaCl) membrane-inserted (*Insertion*) fractions. All subfractions were separated in a 16% Tris-Tricine gel and analyzed by Western blotting with 6E10. *Ctrl*, control.

The effect of a neutralizing pH on Aβ42-induced lysosomal labilization was examined by measuring the release of β-hexosaminidase into the cytosol in differentiated PC12 cells in the presence and absence of 5 nM BafA1. As shown in Fig. 7*B*, the activity of the released β-hexosaminidase in the cytosolic fraction of Aβ42-treated cells was 40% higher than that in the untreated control. BafA1 treatment resulted in a pronounced decrease in Aβ42-induced lysosomal leakage, whereas BafA1 alone did not affect lysosomal leakage of β-hexosaminidase. These results indicate that BafA1 moderates the Aβ42-induced instability of lysosomes.

We also separately examined the effects of Aβ42, Aβ40, and Aβ28 on the latency of isolated lysosome under neutral or acidic pH. As shown in Fig. 7*C*, we observed deleterious effects of Aβ42 on lysosomal membranes that were especially prominent under acidic pH, whereas Aβ40 and Aβ28 did not obviously affect lysosomal membrane intactness. These results consistently demonstrated that Aβ42 caused membrane instability under lower pH.

Alkalizing Drugs Provide Dose-dependent Protection against Aβ42-mediated Cytotoxicity—The effects of BafA1 and chloroquine on Aβ42-induced cytotoxicity were determined using an MTT assay and lactate dehydrogenase release. Fig. 8*A* shows that cell toxicity measured by the MTT assay increased with the concentration of Aβ42, whereas the addition of BafA1 partly prevented cell death induced by Aβ42 at all concentration levels. A similar phenomenon was observed in the lactate dehy-

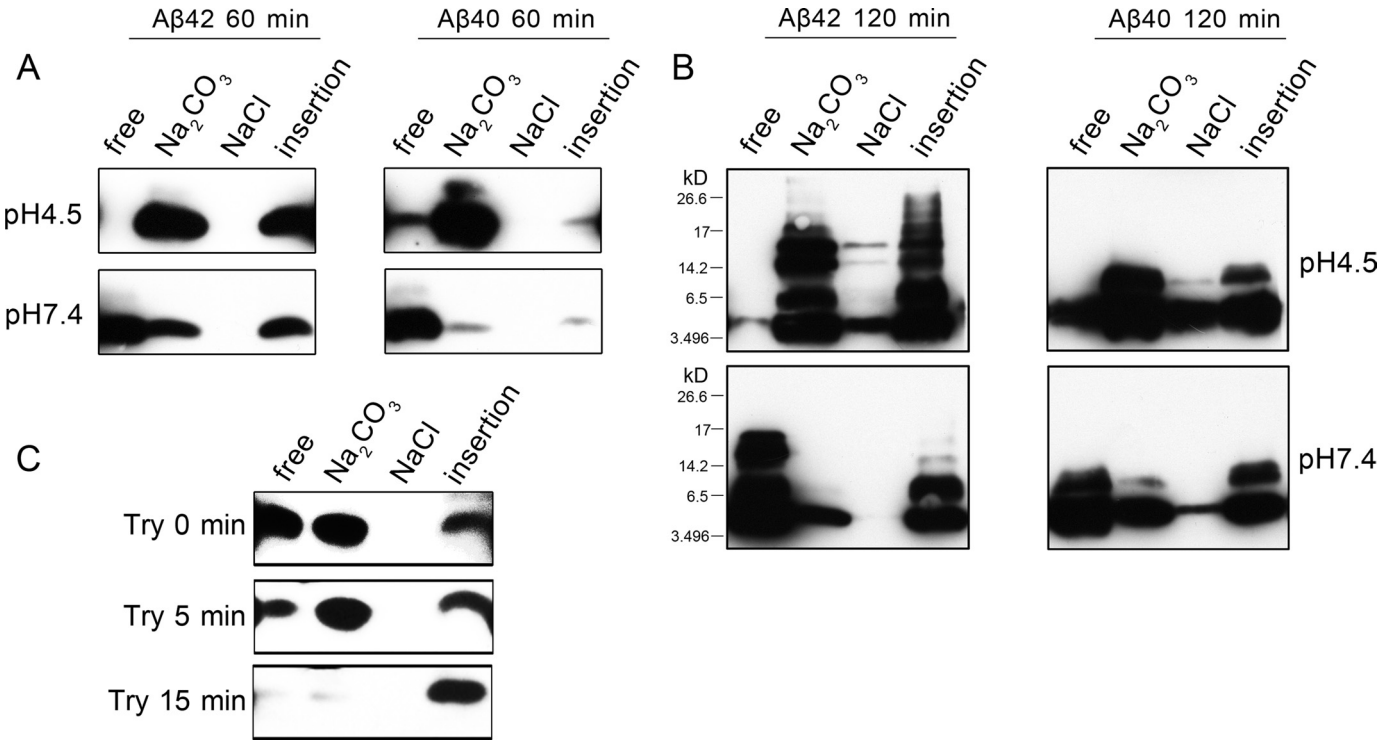

FIGURE 6. **Different effects of pH on the interaction of Aβ42 and Aβ40 with lysosomal membranes.** *A*, samples containing 200 μg of protein of isolated PC12 cell lysosomes resuspended in 200 μl of Na_2HPO_4-citric acid buffers were sonicated on ice for 15 s, and freshly dissolved Aβ peptides (0.1 μM, monomer) were then added and incubated at 37 °C for 60 min. After incubation the samples were fractionated into free Aβ (*free*), 0.1 M carbonate extract (*Na_2CO_3*), 1 M NaCl extract (*NaCl*), and membrane-inserted fraction (*insertion*) as described under "Materials and Methods." *B*, furthermore, samples were prepared after incubation with fresh Aβ42 or Aβ40 for 120 min. *C*, after incubation for 60 min at pH 7.4, a mixture of Aβ42 and membrane was digested with 1 mg/ml trypsin (*Try*) at 37 °C for the indicated times. After digestion, reaction mixtures were ultracentrifuged as above and analyzed by immunoblotting.

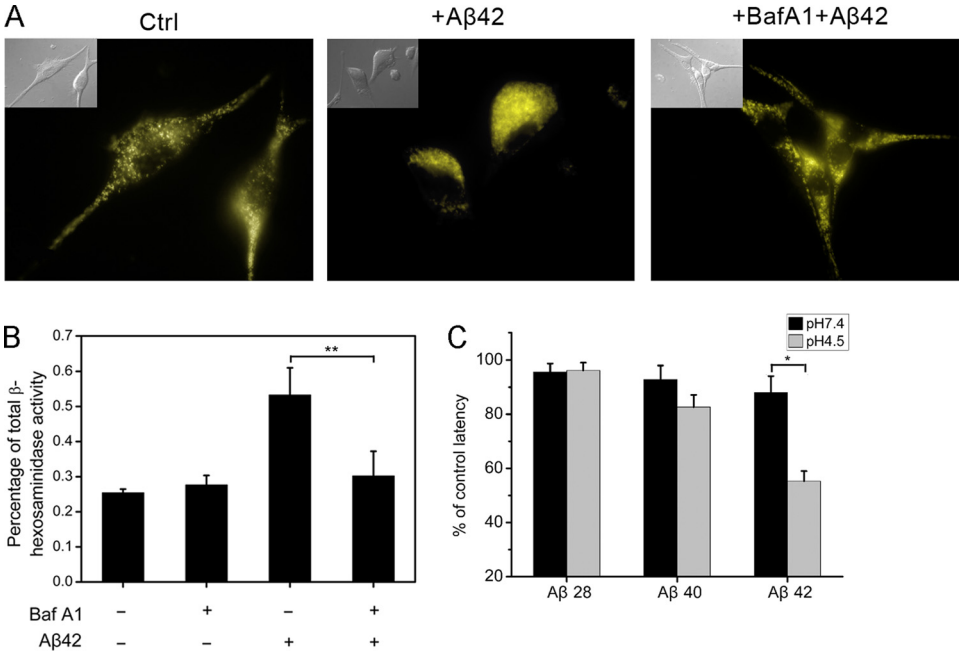

FIGURE 7. **Aβ42-induced lysosomal membrane permeability in a pH-dependent manner.** *A*, BafA1 moderated Aβ42-induced release of Lucifer Yellow from the lysosomes of PC12 cells. Lucifer Yellow-labeled cells were incubated with or without BafA1 (5 nM). After 30 min, 25 μM Aβ42 was added. The cells were then incubated at 37 °C for 6 h and visualized under a Nikon fluorescence microscope. Aβ42 treatment caused lysosomal leakage as revealed by fluorescence that diffused into the cytoplasm; pretreatment with BafA1 moderated the Aβ42-induced release of Lucifer Yellow, and the cells displayed the same discrete punctate pattern of fluorescence as the control (*Ctrl*). The *inset* is the corresponding phase-contrast image. *B*, cytosolic β-hexosaminidase activity was determined as described under "Materials and Methods" to evaluate the effect of BafA1 on Aβ42-induced lysosome leakage. Values are the means ± S.D. of three independent experiments performed in duplicate. Statistical analysis was conducted using Student's two-tailed *t* test (*, $p < 0.05$; **, $p < 0.05$). *C*, shown is the effect of Aβ isoforms on the latency of isolated lysosomes. Purified lysosomes were resuspended in isotonic citrate/phosphate buffer (5 mM, adjusted with sucrose) and incubated with 1 μM Aβ at 37 °C for 30 min before measuring β-hexosaminidase activity in the presence or absence of 0.1% Triton X-100.

drogenase release assay (Fig. 8*B*). The effect of chloroquine on Aβ42-induced cytotoxicity was identical to that of BafA1 (Fig. 8, *C* and *D*). These results suggest that lower pH plays a critical role in Aβ42-induced cell toxicity.

Aβ42 Inserts into Lysosomal Membrane and Induces Lysosome Instability in Aged Transgenic Mice—Finally we investigated whether the insertion of Aβ42 into the lysosomal membrane and the resulting labilization occurred *in vivo*. For this, we isolated lysosomes from cortical regions or hippocampus from brains of transgenic mice at different ages (2 and 10 months). Analogous wild type mice were used as control. In 10-month-old transgenic mice, the immunoblotting of subfractions of cortex lysosomes with an antibody specific to Aβ42 showed that most of the Aβ42 accumulated in lysosomes inserts into the membrane, with a little loosely attached (Fig. 9*A*). As for 2-month-old transgenic mice and wild type mice, the accumulation of Aβ42 in brains is not obvious, and we did not observe similar phenomenon (data not shown).

Next we evaluated the stability of lysosomes in transgenic mice brain by studying the subcellular distribution of the lysosomal matrix enzyme β-hexosaminidase after homogenization and the leakage of this enzyme from the lysosomal fraction after incubation in hypotonic condition. First, we found that the percentage of β-hexosaminidase in the cytosolic fraction showed an apparent increase in aged transgenic mice, with about an 80% increase in the 10-month-old transgenic mice compared with the same old wild type mice (Fig. 9*B*). The increase of free β-hexosaminidase reflects the increase of lysosomal instability in the older transgenic mice brains. The lysosome labilization in aged transgenic mice brain was further established by the evidence that the integrity of the lysosomes from aged transgenic mice decreased greatly after incubation at 37 °C for 5 min in hypotonic sucrose than that of the control lysosomes (Fig. 9*C* and *D*). It indicates that the lysosomes of aged transgenic mice were more susceptible to the osmotic imbalance across their membranes and, therefore, lost their enzyme latency markedly.

DISCUSSION

Although the pathogenic pathway of Aβ-induced neuronal death in AD has been investigated for years, the definitive pathogenesis is still unclear. Recently, a lysosomal branch of the cell death cascade may be important in the disease process. The prevailing model describes that Aβ accumulation in lysosomes resists degradation and eventually causes the release of degradation-resistant insoluble aggregates and other lysosome contents into the cytosol, consequently leading to neuron toxicity (21–23, 61). However, the molecular mechanism involved in the lysosomal accumulation of Aβ42 remains to be clarified. Additionally, lysosomes are organelles that contain a variety of digestive enzymes. Thus, knowing how a mass of Aβ42 escapes degradation and accumulates in lysosomes is important in understanding Aβ42-specific neurotoxicity.

We first employed two model membrane systems, monolayers and vesicles (a closed bilayer), to explore the effect of pH on Aβ42-membrane interactions. We found that Aβ42 could insert into the lipid vesicles via its hydrophobic C terminus under acidic conditions and that the membrane insertion ability of Aβ42 increased with decreasing pH. This implies that Aβ42 can insert into the membranes of endosomes (pH 5–6.5) and lysosomes (pH 4–5). Therefore, we isolated and purified lysosomes from differentiated PC12 cells with extracellularly applied Aβ42 and N2a APPswe×PS1-transfected cells and examined the intralysosomal distribution of exogenous and endogenous Aβ42.

For the first time we determined that lysosomal-accumulated Aβ42 could be divided into three parts: a soluble state in the lysosome lumen; a membrane-attached state, loosely adsorbed onto the lysosomal membrane surface that can be

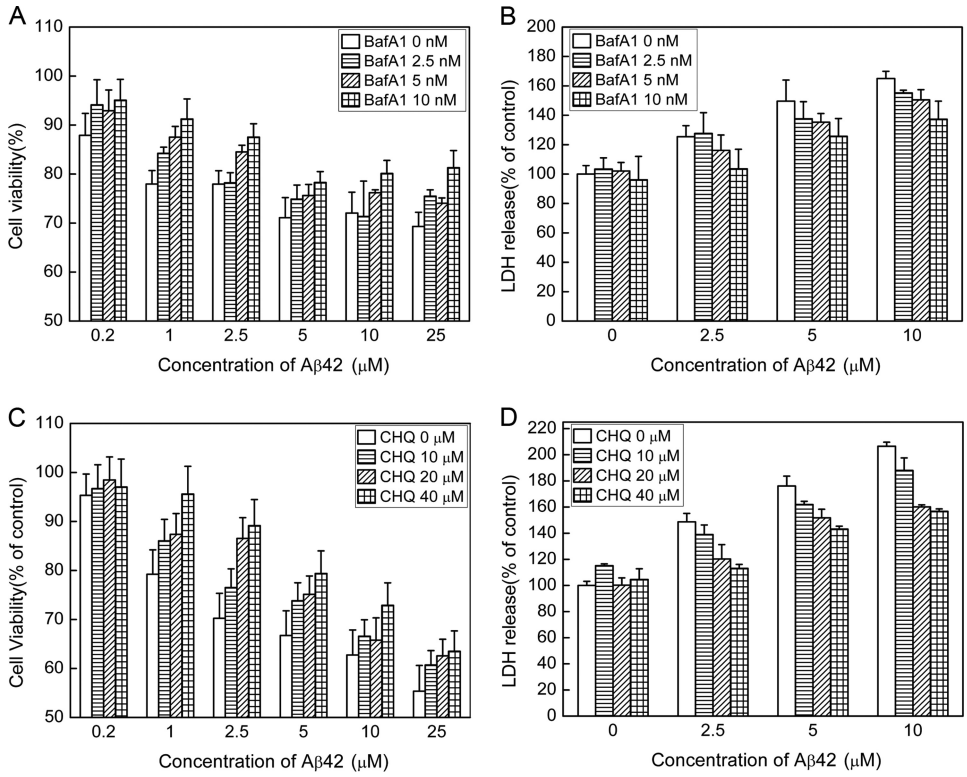

FIGURE 8. **BafA1 and chloroquine moderated Aβ42-mediated cytotoxicity.** Cytotoxicity was determined by a MTT assay (*A* and *C*) or lactate dehydrogenase (*LDH*) release assay (*B* and *D*) as described under "Materials and Methods." Differentiated PC12 cells were preincubated with various doses of BafA1 (*A* and *B*) or chloroquine (*CHQ*, *C* and *D*). Aβ42 was added after 30 min. The cells were then incubated for 48 h, and the assays were performed.

detached by cations; and a membrane-inserted state, tightly associated with the membrane, that cannot be extracted by cations. In differentiated PC12 cells, with the addition of 1 μM Aβ42, we found that a great majority of the lysosomal-accumulated Aβ42 was in a membrane-associated state, whereas the Aβ42 in the lysosomal lumen was minimal and nearly undetectable under the experimental conditions. Moreover, we found that the membrane-related Aβ42 was mostly tightly inserted into the lysosomal membrane, whereas only a small portion was peripherally associated with the membrane. Identical results were obtained from the N2a cells stably expressing human βAPPswe and PS1.

Next, raising the intracellular pH with alkalizing drugs reduced the level of the membrane-inserted Aβ42 and increased that of the membrane-attached portion. Moreover, after prolonging the incubation time of Aβ42 with PC12 cells from 6 to 12 and 24 h, quite a large amount of Aβ42 was found to be retained in the membrane-inserted fraction, whereas the membrane-attached Aβ42 disappeared. Trypsin digestion of the incubation mixture of Aβ42 and the membrane also indicated that the membrane-inserted Aβ42 cannot be easily hydrolyzed. Based on these results, one can reasonably presume that the massive accumulation of Aβ42 in lysosomes may result from nondegradation by lysosomal proteases because of its membrane insertion, thereby providing the physical prerequisite for Aβ42 neurotoxicity.

Our previous study showed that the insertion ability of Aβ40 is strongly dependent upon the ratio of cholesterol to phospholipids under neutral pH (37). However, in a separate examination we found that cholesterol has little influence on the membrane insertion ability of Aβ42; the corresponding πc for eggPC and the cholesterol-containing eggPC monolayer are almost the same (data not shown). Here we show that there are significant differences in the membrane insertion between Aβ42 and Aβ40, and the level of membrane-inserted Aβ40 was very low or negligible compared with Aβ42 at either pH 4.5 or 7.4 (Fig. 6*A*). Our investigation on the hydrophobic exposure of Aβ42 by an 1-anilino-8-naphthalenesulfonate fluorescence probe showed that the hydrophobic exposure of Aβ42 increases with decreasing pH (data not shown), corresponding to its increasing ability of membrane insertion. These data suggested that the magnitude of hydrophobicity may be the main factor involved in the membrane insertion, and the difference in membrane insertion ability between Aβ42 and Aβ40 may be due to the difference of the two hydrophobic amino acid residues, as Aβ28, the isoform without C-terminal transmembrane domain, cannot insert into the membrane at all.

Unlike Aβ42, in cell culture models Aβ40 was degraded much more rapidly after internalization (23, 25), and in AD patients and animal models, it is Aβ42, not Aβ40, that is detected in intracellular accumulation (8, 29, 56, 62). Our comparison of the plasma membrane binding of Aβ in differentiated PC12 cells (supplemental Fig. S6) shows that the binding of Aβ42 is only about 2-fold that of Aβ40. Thus, the great difference in the cumulative amount of internalized Aβ42 and Aβ40 may be caused mainly by the different rate of degradation due to their difference in membrane insertion ability. Additionally, incubation of Aβ with the lysosomal membrane showed that membrane-inserted Aβ42 tends to form multi-oligomeric aggregates that are poorly degraded, whereas Aβ40 forms only dimers. Thus, this differential interaction of Aβ42 and Aβ40 with the membrane could be the underlying cause for the preferential accumulation of Aβ42 in lysosomes and neuronal death. This finding may explain the paradoxical observations that Aβ40 is the primary proteolytic product of APP in neurons (63), whereas it is Aβ42 that predominantly exhibits intraneuronal accumulation and neurotoxicity (64, 65).

However, our present study does not account for the formation of Aβ oligomers and the particular mechanism of membrane insertion of the protein. From the *in vitro* interaction results of Aβ with membrane in this current manuscript (Fig. 6*A*), we can make sure that monomeric Aβ can insert into the

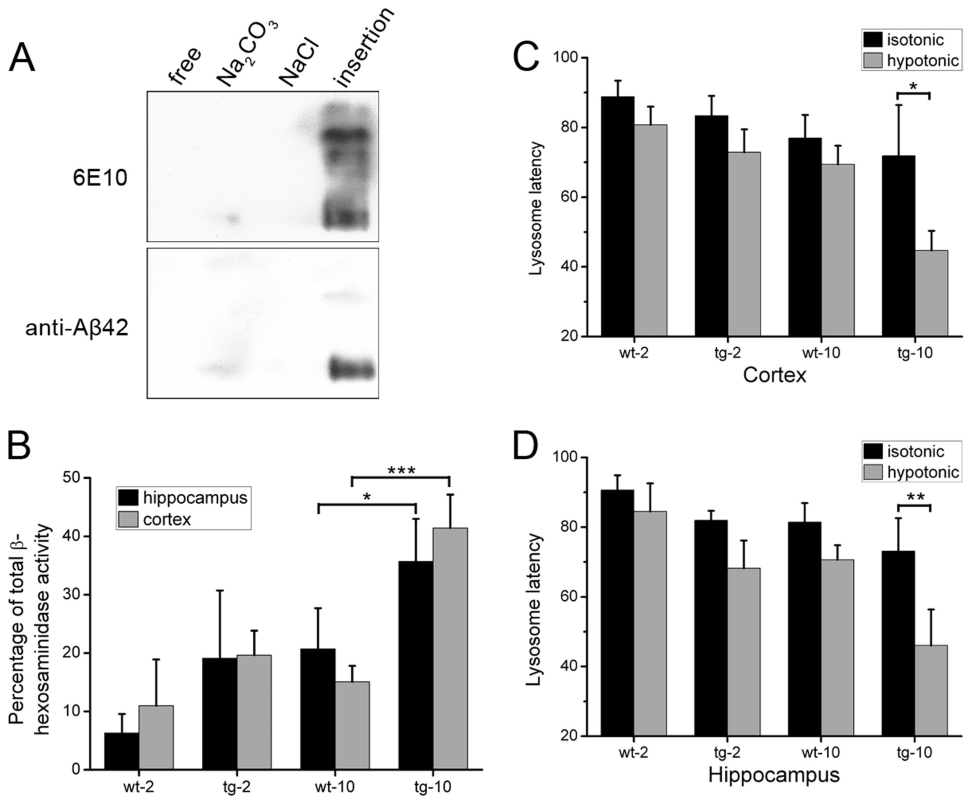

FIGURE 9. **Aβ42 inserts into lysosomal membrane and induces lysosome instability in aged transgenic mice.** *A*, the distribution of Aβ42 in lysosomes from cortex of 10-month-old APP×PS1 transgenic (*tg-10*) mice was examined in each lysosomal fraction using specific anti-Aβ42 and 6E10 antibodies. *wt*, wild type, *B*, β-hexosaminidase activity in the cytosolic fraction is plotted as the percentage of total activity. Homogenate from each group ($n = 4$ mice per group) was separated into cytosolic and pellet fractions. β-Hexosaminidase activity in each fraction was measured (*, $p < 0.05$; ***, $p < 0.001$). Shown are the effects of hypotonic conditions on the osmotic stability of cortical (*C*) or hippocampal (*D*) lysosomes from APP×PS1 transgenic or wild type mice at different ages. Lysosomes were incubated in isotonic (0.25 M) or hypotonic (0.15 M) sucrose medium at 37 °C for 5 min. Free and total β-hexosaminidase activity was measured immediately after the incubation. Lysosome latency was calculated. Values are the means ± S.D. of four measurements (*, $p < 0.05$; **, $p < 0.05$).

membrane. There may be many aggregates in solution for the aggregating property of Aβ42. Aβ oligomerization has also been shown to occur during interaction with lipid bilayers, in particular lipid rafts (66, 67). Therefore, we cannot distinguish the origination of the oligomeric Aβ42 on the membrane. It is unclear whether the membrane insertion of Aβ42 takes place before, after, or during its oligomerization. The mechanism and dynamics by which Aβ42 oligomerizes when it interacts with membrane are very complicated and remain to be established.

Previous reports have suggested that the lysosomal accumulation of Aβ42 may play an important role in neuronal cell death and that the loss of lysosomal membrane impermeability is involved in Aβ42 pathogenesis (22, 23, 68, 69), but what is essential for the Aβ42-induced lysosomal damage remains to be shown. We confirmed the view that Aβ42 applied extracellularly leads to fluorescence diffusion of endocytosed punctate Lucifer Yellow and lysosomal enzyme leakage. Treatment with alkalizing drugs, such as chloroquine and BafA1, obviously ameliorated lysosome damage and cell death. A recent publication by Ji *et al.* (68, 69) reported that apoE4 and Aβ42 might work in concert at lower pH to increase the susceptibility of lysosomes to disruption. They also pointed out that the question of whether the apoE4/Aβ42 complex or the two molecules, independently, destabilize the membranes still remains to be resolved. Our study demonstrates that Aβ42 itself in the membrane-inserted form can destabilize the lysosomal membrane. Consistent with the observations that Aβ42 inserts to the lysosomal membrane at acidic pH and destabilizes them *in vitro*, aged transgenic mice expressing human APP and PS1 also showed tight binding of Aβ42 to the lysosomal membrane in the cortical region and fragile lysosomes in cortex and hippocampus. These data *in vivo* emphasized the importance of lysosomal pathway induced by Aβ42.

Taken together, if Aβ42 resulting from extracellular uptake or intracellular generation accumulates within the endocytic compartments of neurons, the acidic characteristics of endosomal/lysosomal organelles could provide a favorable environment for the membrane insertion of Aβ42 that may protect it from degradation and result in lysosome instability. Additionally, the concentration effect of lysosomes and the pathological accumulation of membrane-bound Aβ42 may favor the oligomerization of Aβ42, which probably then acts as a seed for further aggregation. Our findings suggest that lysosomal Aβ insertion may be pathogenic, and regulating intracellular pH pharmacologically may have a therapeutic effect on AD.

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Supplemental Information

Routine transmission electron microscopy and immunoglod labeling of ultrathin cryosections

The isolated lysosomal fraction was fixed for 2 h at 0°C with 2% paraformaldehyde, 0.5% glutaraldehyde in 0.25 M sucrose solution adjusted to pH 7.4 with 50 mM phosphate buffer (PB). After fixation, the sample was thoroughly washed several times with 0.1 M PB (pH 7.4) and then post-fixed for 1 h with 1 % osmium tetroxide. The sample was subsequently rinsed in distilled water, dehydrated with an ascending series of ethanol and embedded in Epon 812. Ultrathin sections were prepared with a diamond knife and mounted on Formvar-coated grids. They were stained with saturated uranyl acetate for 30 min, followed by lead citrate for 20 min, and examined with a Philips CM120 transmission electron microscopy. For immunogold labeling, the grid was labeled with Lamp-1 primary antibody and gold-labled secondary antibody before uranyl and lead staining.

Immunogold electron microscopy of transgenic mice brain

Double APP×PS1 transgenic mice used in this study were from Institute of laboratory animalsciences, CAMS&PUMC and handled in accordance with the Chinese guidelines for animal welfare and experimental protocol. Mice were anesthetized with aether and perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were removed and post-fixed for 1 h in 2.5% glutaraldehyde at 4°C. One-mm3 blocks were excised from hippocampus, fixed in 1% osmium tetroxide in PBS, embedded in LR White, sectioned (80 nm), double immunolabeled with anti-Aβ42 and anti-Lamp-1, and stained with uranyl acetate and lead citrate. The final preparations were examined with a Philips CM120 transmission electron microscopy.

Immunoelectron microscopy of Aβ42

After 60 min incubation of Aβ42 with purified lysosomal membrane, mixture was ultracentrifuged to remove free Aβ42, and washed by 0.1 M Na_2CO_3 and 1 M NaCl to remove adsorbed Aβ42. The ultimate membrane pellet was suspended in PBS, put on Formvar-coated electron microscope grids, fixed and immuno-labeled with the specific N-terminus antibody 6E10 and C-terminus antibody of Aβ42.

Cell surface binding of Aβ

NGF-differentiated PC12 cells were incubated for 2 h at 4°C in medium containing different concentration of Aβ42 and Aβ40, and washed twice with cold PBS after incubation. The amount of cell-associated Aβ was determined by Elisa or by Western blot.

Table s1: MS Results of hydrolysis of Aβ42 by papain. The hydrolysis lasted for 30 min after the addition of papain into the Aβ42 solution and iodoacetamide was then injected to end the reaction. The mixture was detected by MS after mixing with the matrix-CCA.

number	fragment	$M+H^+$ (m/z)	
		Calculated	Measured
1	^{1}DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA^{42}	4512.3*	4512.4
2	^{12}VHHQKLVF^{19}	1029.6 (+Na)	1029.7
3	^{1}DAEFRHDSGYE^{11}	1325.5	1325.6
4	^{1}DAEFRHDSGYEVH^{13}	1561.7	1561.7
5	^{1}DAEFRHDSGYEVHHQK^{16}	1954.9	1955.0
6	^{12}VHHQKLVFFAEDVGSNK^{28}	1955.0	1955.0
7	^{22}EDVGSNKGAIIGLMVGGVVIA^{42}	2037.1(+Na)$^{\#}$	2037.0
8	^{6}HDSGYEVHHQKLVFFAEDVG^{25}	2314.1	2314.3
9	^{1}DAEFRHDSGYEVHHQKLVF^{19}	2314.1	2314.3
10	^{1}DAEFRHDSGYEVHHQKLVFF^{20}	2461.2	2461.4
11	^{1}DAEFRHDSGYEVHHQKLVFFA^{21}	2532.2	2532.4
12	^{11}EVHHQKLVFFAEDVGSNKGAIIGLMVG^{37}	2933.5(+Na)$^{\#}$	2933.3
13	^{1}DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIG^{33}	3672.8	3672.9
14	^{1}DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG^{37}	4073.0	4072.9

*: molecular ion

#: Met is oxidized

Many peaks in the spectra show an increase of 22 caused by the addition of Na^+ to the MALDI ion.

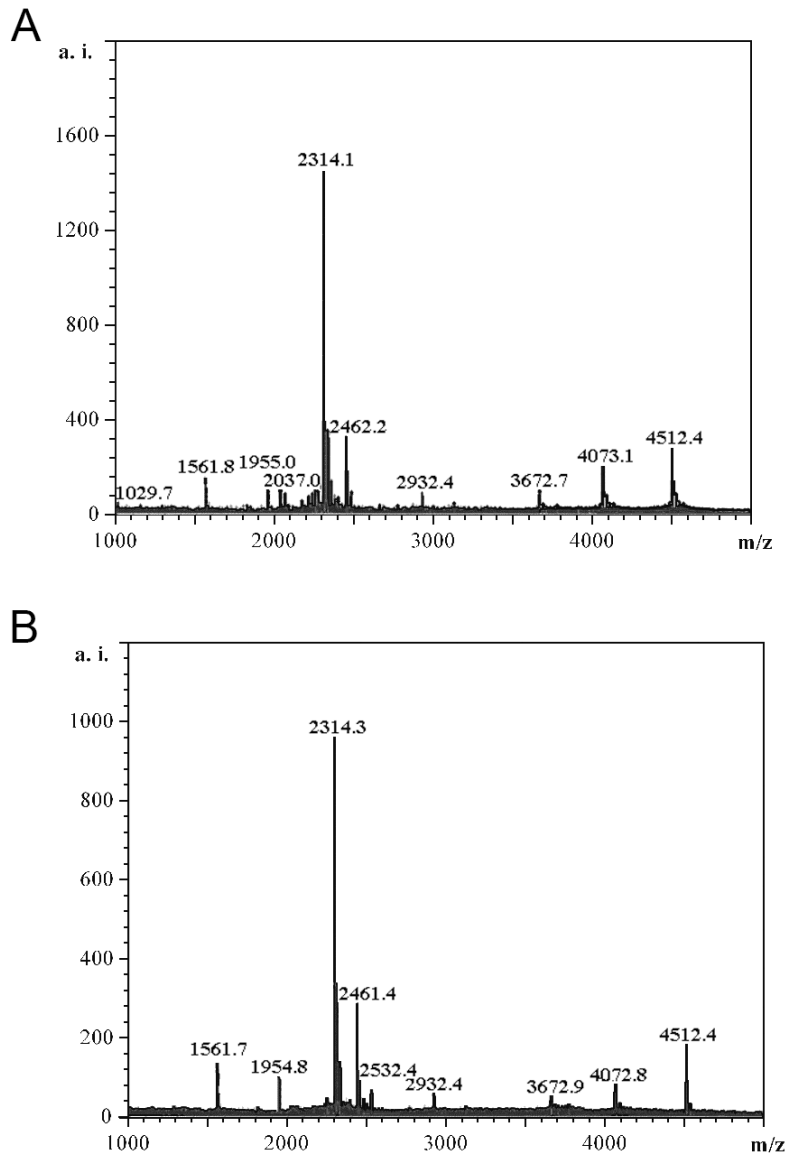

Figure S1. The hydrolysis MS spectra of Aβ42 at pH 7.0. MS spectra of Aβ42 in 5 mM Na_2HPO_4-HAc buffer only (A) or in the presence of eggPC vesicles (B) were obtained after papain hydrolysis for 30 min at 37°C and a peptide/enzyme ratio of 20 (mol/mol).

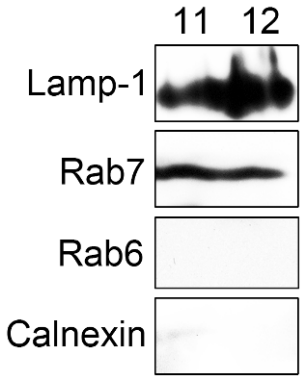

Figure S2. Western blotting analysis of lysosomal fractions. Proteins from lysosomal fractions were separated on a 12% SDS-PAGE gel and probed with the indicated antibodies.

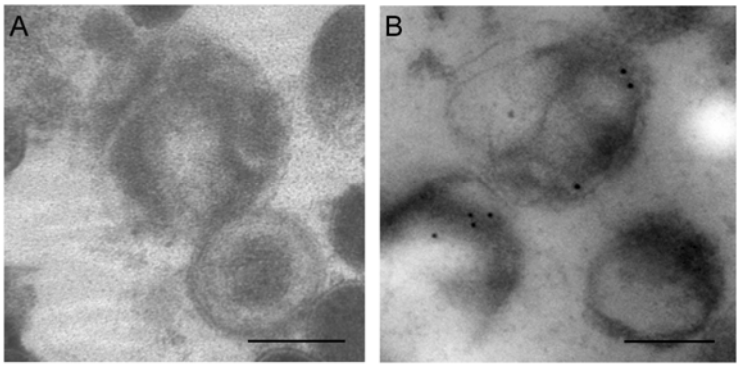

Figure S3. Routine transmission electron micrograph of the purified lysosomal fraction. (A) Control without primary antibody. (B) Immunoelectron microscopy labeled with Lamp-1. Gold: 10 nm. Scale bars: 200 nm.

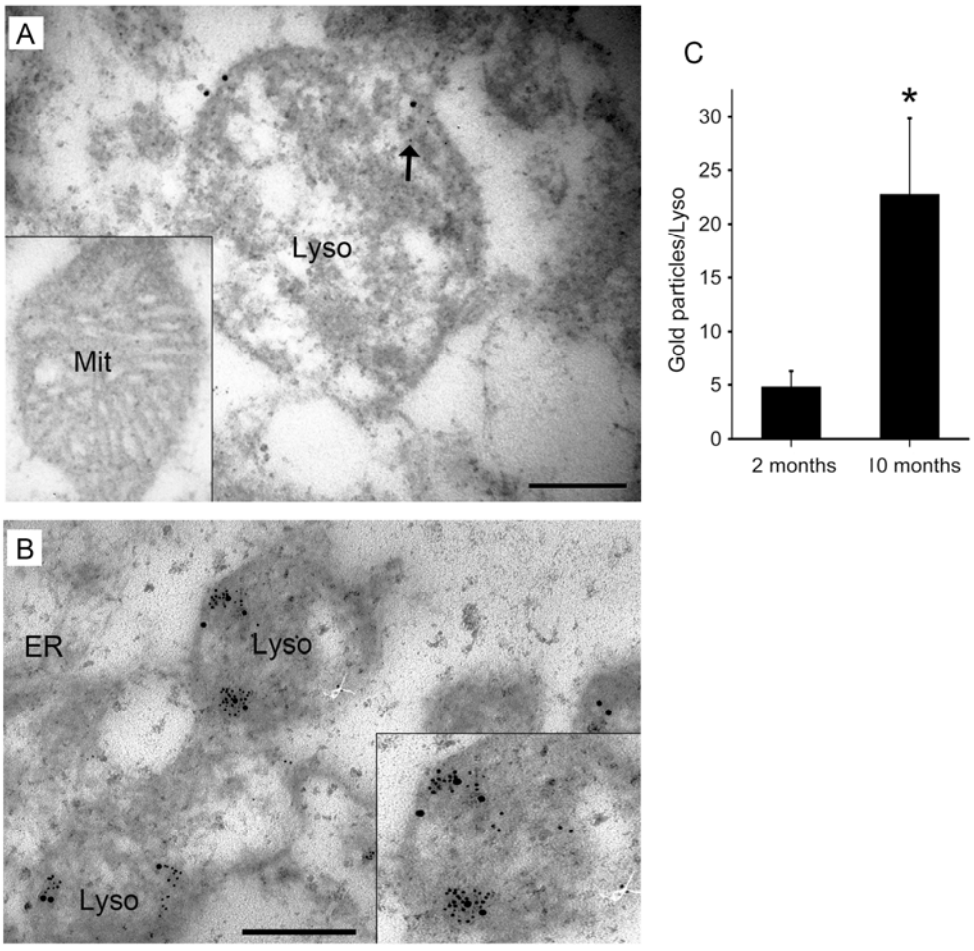

Figure S4. Immunoelectron microscopy of hippocampus from APPswe×PS1 transgenic mice. (A) Double immunolabeling of a lysosome from young (2 months) transgenic mouse with anti-Aβ42 (6-nm gold particle, arrow) and anti-Lamp-1 (12-nm gold particle). Inset is representative mitochondria. (B) Double immunolableing of intraneuronal vesicles from older (10 months) transgenic mouse with anti-Aβ42 (6-nm gold particle) and anti-Lamp-1 (12-nm gold particle). Inset is a higher magnification of the Aβ42 associated lysosome. (C) Bar graph indicating the number of gold particles per lysosome in 2-month-old and 10-month-old transgenic mice (Asterisk denotes statistical significance). Abbreviations: Lyso, lysosome;

Mit: mitochondria; ER: endoplasmic reticulum. Scale bars: 200 nm.

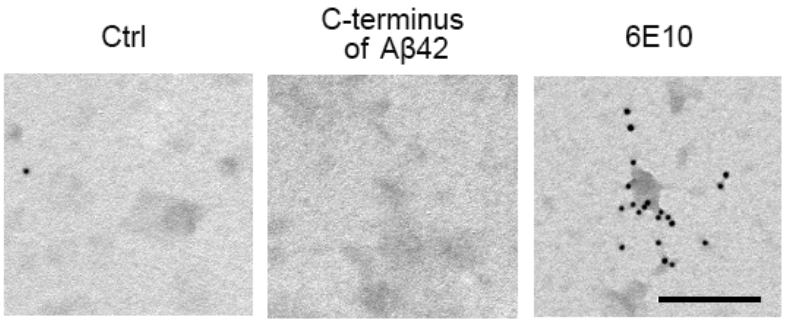

Figure S5. Immunoelectron microscopy of Aβ42 inserted into the lysosomal membrane. Samples were immuno-gold labeled with an Aβ42 C-terminus antibody or N-terminus antibody 6E10, or without a primary antibody (Ctrl). Gold: 10 nm. Scale bar: 200 nm.

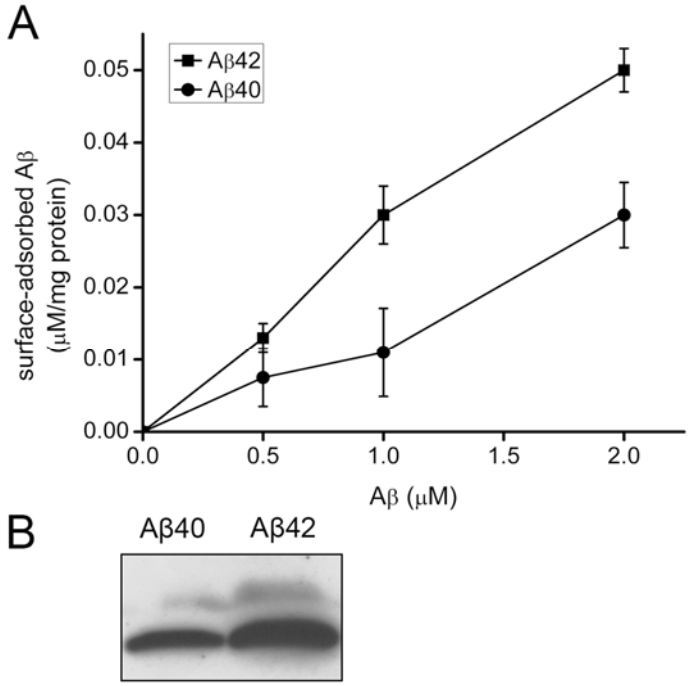

Figure S6. Cell surface adsorption of Aβ to differentiated PC12 cells at 4°C. (A) Adsorption analysis of Aβ by Elisa assay. Differentiated PC12 cells were incubated for 2 h at 4°C in medium containing the indicated concentrations of Aβ42 and Aβ40, washed twice with cold PBS and the amount of cell-associated Aβ was determined by Elisa. Values are the means ± S.D. of two experiments. (B) Adsorption analysis of 2 μM Aβ42 and Aβ40 to differentiated PC12 cells by Western blot with 6E10 antibody.

Neurobiology:

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