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The Presence of Amyloid β-Protein in the Detergent-Insoluble Membrane Compartment of Human Neuroblastoma Cells†

Maho Morishima-Kawashima‡ and Yasuo Ihara*,‡,§

Department of Neuropathology, Faculty of Medicine, University of Tokyo, Tokyo, Japan, and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Saitama, Japan

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ABSTRACT: To investigate the intracellular compartmentalization of amyloid β-protein (Aβ), human neuroblastoma SH-SY5Y cells were fractionated and the Aβ content in each fraction was quantitated by the well-characterized two-site enzyme-linked immunosorbent assay (ELISA). Subcellular fractionation of the cell revealed two distinct pools of Aβ within the cells: a Triton-soluble and a Triton-insoluble pools with the latter being larger than the former. Because Triton insolubility points to caveolae-like domains, we prepared detergent-insoluble, low-density membrane domains from SH-SY5Y cells using two different protocols. The low-density membrane fraction prepared by either protocol was found to contain a substantial proportion of intracellular Aβ40 and Aβ42. These results indicate that the distinct membrane domains are involved in the generation and/or trafficking of Aβ.

Alzheimer's disease (AD)[1] is a multigenic disorder leading to the common pathological alterations: formation of senile plaques and neurofibrillary tangles throughout the cortex, followed by extensive loss of subsets of neurons. The major component of senile plaques is amyloid β-protein (Aβ), a small protein with $M_r \sim 4000$, which is derived from a much larger membrane protein, designated as β-amyloid precursor protein (APP). Aβ has been intensively focused on in AD research, because (i) all familial AD-linked mutations, namely mutations of the APP, presenilin 1, and 2 genes, cause an increased secretion of Aβ42, a longer form of Aβ (*1*); (ii) Down syndrome patients who invariably develop AD pathology in middle life contain increased levels of Aβ42 in the plasma (*2*); (iii) ApoE4, a strong risk factor for AD, is reported to accelerate the deposition of Aβ in the brain (*3*); and finally, (iv) Aβ42, the more amyloidogenic species, appears to be initially deposited in AD brains (*4*).

Although Aβ42 is the predominant species of senile plaques, Aβ40 is the major species normally secreted from cells. It is claimed that Aβ42 is generated in the ER/intermediate compartment (*5*−*7*), while Aβ40 is presumed to be generated in the trans Golgi network (*5*, *8*). An endocytic pathway may also be involved in the generation of Aβ (*9*). However, the precise mechanism of Aβ cleavage or intracellular trafficking of Aβ remains largely unknown.

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* Address correspondence to Yasuo lhara, MD, Department of Neuropathology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Tel: +81-3-3812-2111, ext 3541. Fax: +81-3-5800−6852. E-mail: yihara@m.u-tokyo.ac.jp.

‡ University of Tokyo.

§ Japan Science and Technology Corporation.

[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; APP, β-amyloid precursor protein; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; DIG, detergent-insoluble, glycolipid-enriched domain; MBS, MES-buffered saline; PBS, phosphate-buffered saline.

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We have recently found that Aβ already accumulates to a significant extent in the normal aged brain in which Aβ deposition cannot be detected using immunocytochemistry (*10*, *11*). This may raise the question of whether normally secreted Aβ is involved at all at the initial stage of Aβ deposition. Furthermore, GM1 ganglioside-bound Aβ is detected in brains exclusively showing diffuse plaques, the earliest stage of senile plaques (*12*). This suggests that the Aβ that is located in GM1-enriched membrane domains within the cell may be shed into the extracellular space, thereby acting as a seed for amyloid formation (*12*). This speculation was validated by an in vitro experiment: the addition of GM1-containing vesicles to Aβ solution dramatically accelerates the rate of Aβ fibril formation (*13*). Thus, these findings suggest the possibility that the earliest abnormality in β-amyloidogenesis is presumably in the intracellular trafficking of the specific membrane domains accompanying Aβ. In this study we sought to characterize intracellular compartmentalization of Aβ in untransfected human neuroblastoma SH-SY5Y cells using two-site ELISA.

EXPERIMENTAL PROCEDURES

Antibodies. The antibodies used for ELISA were BAN50 (raised against Aβ1−16; the epitope is located in Aβ1−10), BNT77 (raised against Aβ11−28; the epitope is located in Aβ11−16), BA27 (raised against Aβ1−40; specific for Aβ40), and BC05 (raised against Aβ35−43; specific for Aβ42). The specificity of these antibodies was previously described in detail (*14*, *15*). 4G8 (specific for Aβ17−24) and 6E10 (raised against Aβ1−17) were obtained from Senetek PLC (Maryland Heights, MO). Polyclonal antibodies against APP were raised against the synthetic peptides, APP666−695 (cytoplasmic domain, APPc) and APP571−580. Monoclonal antibodies against APP (5A3/1G7) (*16*) were provided by Dr. E. H. Koo. Other antibodies used in this study were anti-caveolin, anti-flotillin, anti-fyn, anti-Gβ (Transduction Laboratories, Lexington, KY), and anti-src (Upstate Biotechnology, Lake Plasid, NY).

ELISA. Two-site ELISA for Aβ was carried out as previously described (*10*). BAN50 or BNT77 was coated as the capture antibody, while BA27 or BC05 was used as the detection antibody following conjugation with horseradish peroxidase.

Cell Culture. The human neuroblastoma cell line, SH-SY5Y, was grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12, 1:1 mixture) supplemented with nonessential amino acids (Eagle's formulation) and 10% fetal calf serum (all from Gibco BRL). The cells were plated onto culture dishes coated with collagen Type I.

Subcellular Fractionation. SH-SY5Y cells were washed twice with ice-cold PBS and then collected by centrifugation. The cell pellet was homogenized in 4 volumes of Tris-saline (50 mM Tris-HCl, pH 7.6, 0.15 M NaCl) containing 1% Triton X-100 and various protease inhibitors as described before (*11*), and the homogenates were centrifuged at 540000*g* for 20 min in a TLX ultracentrifuge (Beckman, Palo Alto, CA). The supernatant was subjected to ELISA for the quantitation of Triton-soluble Aβ. The pellet was washed with the same buffer and then resuspended by brief sonication in an initial volume of 6 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.6. The homogenate was centrifuged at 265000*g* for 20 min, and the resultant supernatant was diluted at 1:12, to reduce the concentration of guanidine hydrochloride to 0.5 M (*17*), and subjected to ELISA.

Purification of Detergent-insoluble, Low-Density Membrane Domains. Detergent-insoluble membrane fractions were obtained according to an established method (*18*) with minor modifications. Briefly, SH-SY5Y cells were washed twice with ice-cold PBS, and harvested by centrifugation at 1000*g* for 5 min. The cell pellet was homogenized in MES-buffered saline (25 mM MES, pH 6.5, 0.15 M NaCl; MBS) containing 1% Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, 1 μg/mL pepstatin, and 10 μg/mL aprotinin. After treatment with DNase I (10 units/mL) at 0 °C for 1 h, the extract was adjusted to 40% sucrose by the addition of 2 mL of 80% sucrose in MBS, placed at the bottom of an ultracentrifuge tube, and overlaid with a 5%/35% discontinuous sucrose gradient in MBS without Triton X-100 (4 mL of 5% sucrose/4 mL of 35% sucrose). The gradients were centrifuged at 39 000 rpm for 18−20 h in an SW 41 rotor (Beckman, Palo Alto, CA). A light-scattering interface at 5%/35% sucrose was carefully aspirated, diluted 3-fold with MBS, and centrifuged. The resultant pellet was extracted with guanidine hydrochloride and subjected to ELISA as described above. When this protocol was applied to NIH-3T3 cells, this particular fraction exhibited an opaque band containing caveolin, but excluded most other cellular proteins.

Detergent-free purification of the low-density membrane domain was carried out as described by Song et al. (*19*) using 0.5 M sodium carbonate, pH 11.0.

Immunoprecipitation and Immunoaffinity Chromatography. To deplete APP (and its carboxy-terminal fragments) from the Triton-soluble fraction, ligand-purified anti-APPc was conjugated with beads using ImmunoPure Protein A IgG Orientation kit (Pierce, Rockford, IL). The Triton-soluble fraction from SH-SY5Y cells was applied onto the column, and Aβ and APP levels in the flow-through fraction were quantitated.

The guanidine hydrochloride extract of the Triton-insoluble residue from SH-SY5Y cells was dialyzed against 0.5 M guanidine hydrochloride in 20 mM Tris-HCl, pH 7.6. After brief centrifugation, the supernatants (and Triton-soluble fractions) were precleared with protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and then incubated with BAN50 overnight at 4 °C. The resultant produced immune complexes were isolated with protein G-Sepharose and subjected to Western blotting.

6E10 was conjugated with Affi-Gel Hz Hydrazide gel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. The guanidine hydrochloride extract, after being dialyzed against 0.5 M guanidine hydrochloride in 20 mM Tris-HCl, pH 7.6, was applied onto a 6E10-immobilized column. The bound proteins were eluted from the column with 1% SDS in 20 mM Tris-HCl, pH 6.8.

Western Blotting and Other Methods. The proteins separated on SDS−PAGE were transferred onto a PVDF (polyvinylidenedifluoride; Immobilon; Nihon Millipore Ltd, Yonezawa, Japan) membrane and subjected to Western blotting. Western blotting for Aβ was performed as described previously (*11*). After electrotransfer, a nitrocellulose

membrane (pore size 0.22 μm, Schleicher and Schuell, Germany) was boiled in PBS. The bound antibodies were detected by enhanced chemiluminescence (ECL; Amersham, Buckingham, U.K.).

Quantitation of ECL bands of interest was performed with a model GS-700 imaging densitometer on Molecular Analyst Software (Bio-Rad Laboratories). APP purified from *Escherichia coli* as a fusion gene product (a gift of Dr. T. Kitamoto) was used as the authentic control.

The cellular protein content was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce) in the presence of 1% SDS.

RESULTS

Evaluation of the Specificity of ELISA for Quantitation of Aβ in the Detergent-Soluble Fraction. In the course of our work on the determination of Aβ concentrations within the cells, we became concerned about the possibility that the two-site ELISA for Aβ may detect full-length APP and its carboxy-terminal fragments including the full Aβ sequence. This is because BAN50, BNT77, and BC05 are considered to be sequence-specific; in particular, BC05 is raised against Aβ35−43 and has a weak affinity for Aβ43, although ~50-fold less than that for Aβ42 (*20*). In contrast, BA27, raised against Aβ1−40, has negligible affinity for either Aβ42 or 43 and thus may be end-specific (*14*). If this were the case, then quantitation of intracellular Aβ, particularly of Aβ42, by ELISA would be problematic, because the cell lysate may contain very low levels of Aβ and high levels of APP.

We therefore investigated whether the ELISA detects APP, and if so, to what extent it crossreacts with APP. APP was purified from *E. coli* as a fusion gene product and applied to ELISA, using synthetic Aβ1−40 or Aβ1−42 as a standard. As shown in Figure 1, while BA27 was truly end-specific for Aβ40, BC05 showed a certain, but significant, extent of cross-reactivity with APP in both BAN50- and BNT77-based ELISA (Figure 1A−D). Western blotting provided similar results: All of the Aβ monoclonal antibodies except BA27 crossreacted with APP (Figure 1E). The same cross-reactivities were observed with native APP partially purified from SH-SY5Y cells (data not shown).

The affinity of BC05 for APP was 300~500-fold less than that for Aβ42 (Figure 1, B and D). However, the concentrations of intracellular APP, as assessed by Western blotting, were ~500-fold higher than those of Aβ42 (data not shown). Thus, the ELISA presumably overestimates Aβ42 in the detergent-soluble fraction of the cell lysate. To assess the extent of overestimation by ELISA, the Triton-soluble fraction from SH-SY5Y cells was applied onto an anti-APP immunoaffinity column. When the APP level was depleted up to ~80%, the apparent level of Aβ42 was reduced by more than 60% (Figure 2). This clearly indicates that the Aβ42 in the detergent-soluble fraction of the cell lysate is overestimated by the ELISA; nevertheless, the ELISA value partially [at least 20%, when calculated from observed APP concentrations (data not shown)] represents the true Aβ levels even in the presence of higher concentrations of APP.

Aβ Is Present in Both Triton-Insoluble and Triton-Soluble Fractions. Previous studies on intracellular Aβ have focused on the Aβ in the nonionic detergent-soluble fractions from the cell (*6*, *7*, *21*). In contrast, we have been particularly interested in the Aβ in the detergent-insoluble fractions, because a possible preamyloid form, GM1 ganglioside-bound Aβ, is insoluble in Triton X-100 (*12*). SH-SY5Y cells were extracted with 1% Triton X-100, and the remaining insoluble residues were solubilized with 6 M guanidine hydrochloride. Although highly insoluble Aβ deposits in the brain can be effectively solubilized with formic acid, in our hands guanidine hydrochloride was found to be more effective for extracting Aβ from the detergent-insoluble fraction of the cells than formic acid (data not shown). This observation may suggest that the detergent-insoluble Aβ within the cells is not in the fibrillar form but in another as yet unknown form. As shown in Table 1, significant amounts of Aβ were consistently detected by ELISA in the Triton-insoluble fraction. Notably, the levels of Aβ40 or Aβ42 in the detergent-insoluble fraction appeared to be higher than those in the detergent-soluble fraction. Since APP was undetectable in the Triton-insoluble fraction after extensive extraction with Triton X-100 (data not shown), the ELISA should be considered as enabling accurate quantitation of the Aβ levels in this fraction. Together with the above finding on overestimation, the difference in the levels of Aβ42 between Triton-soluble and Triton-insoluble fractions would become even larger: Thus there are large pools of Aβ40 and Aβ42 in the Triton-insoluble compartment of SH-SY5Y cells.

To rule out the possibility that secreted Aβ is trapped in the plasma membrane, thereby providing false levels of intracellular Aβ, SH-SY5Y cells were treated with trypsin (0.5 mg/mL) before harvesting (*21*). However, this treatment had no effect on the levels of Aβ in the Triton-soluble or Triton-insoluble fraction (data not shown).

To further confirm the presence of Aβ in the Triton-soluble and -insoluble fractions, immunoprecipitation was performed using BAN50, followed by sensitive Western blotting with 6E10. To evaluate the efficiency of immunoprecipitation, 50 pM synthetic Aβ was immunoprecipitated in the same manner. As expected, Western blots of the immunoprecipitates from both fractions clearly showed the Aβ band at ~4 kDa, comigrating with synthetic Aβ (Figure 3A). This result again clearly indicates that both Triton-soluble and -insoluble fractions contain similar levels of Aβ (Aβ40 + Aβ42), although the amount of Aβ recovered from the Triton-insoluble fraction was less than that expected from the ELISA value. Using a 6E10-immobilized column, we confirmed the presence of both Aβ40 and Aβ42 in the Triton-insoluble fraction, although the Aβ42 band was very faint in this experiment (Figure 3B). Thus, these results clearly indicate the presence of both Aβ40 and Aβ42 in the Triton-insoluble compartment within the neuroblastoma cells.

Furthermore, the aforementioned procedure revealed a distinct 6E10- and BA27-immunoreactive band at 7~8 kDa in addition to the Aβ band at ~4 kDa in the Triton-insoluble fraction (Figure 3B). Because BA27 is truly end-specific for Aβ40, the result indicates that at least a substantial proportion of the particular protein terminates at Val-40, and its faint BC05 reactivity suggests that a much smaller proportion probably terminates at Ala-42 (Figure 3B). Thus, it is highly likely that the Aβ-immunoreactive protein at 7~8 kDa undergoes γ-cleavage. One possibility would be that this particular protein is an N-terminally extended one containing the full-length Aβ sequence. To examine this

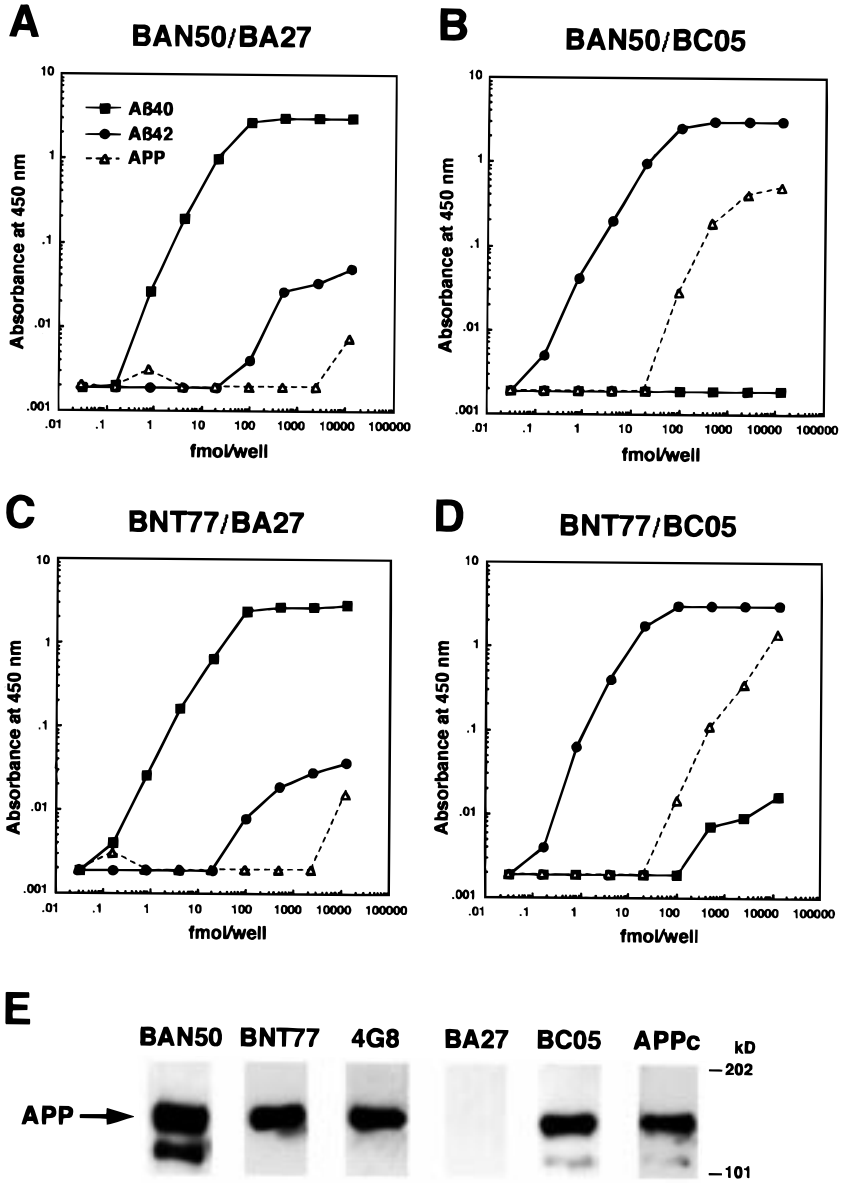

FIGURE 1: Specificities of ELISA and Aβ monoclonal antibodies. The specificities of ELISA for Aβ40 and Aβ42 (A−D) and Aβ monoclonal antibodies (E) were assessed as described under Experimental Procedures. (A−D) Aβ40 is indicated by ■, Aβ42 is by ●, and APP is by △. (E) APP purified from *E. coli* was subjected to SDS−PAGE followed by Western blotting with various antibodies against Aβ (BAN50, BNT77, 4G8, BA27, and BC05) and APP (APPc). BA27 showed no cross-reactivity with APP even after a longer exposure. A lower band seen in some blots presumably represents a degradative product of APP.

possibility, we raised an antibody against APP571−580 which is located 17−26 residues away from the N-terminus of Aβ. Although this antibody recognized the antigen peptide by ELISA and APP by Western blotting, it did not label the 7∼8 kDa band on the blot (data not shown).

Intracellular Aβ Associated with Detergent-Insoluble, Low-Density Membrane Domains. One of the characteristic compartments exhibiting Triton insolubility is a detergent-insoluble, glycolipid-enriched domain (DIG) (for reviews see refs *22* and *23*). DIGs are rich in glycosphingolipid and cholesterol and appear to be involved in vesicular trafficking and signal transduction. Caveolae are a morphological counterpart of DIGs. Because of their unusual lipid composition, DIGs can be readily purified as a low-buoyant density fraction.

To clarify whether the Triton-insoluble Aβ indicates its association with the specific membrane domain, detergent-insoluble, low-density membrane fractions were prepared from SH-SY5Y cells by sucrose density gradient centrifugation. Although caveolin, a marker protein of caveolae, was undetectable in neuroblastoma cells, flotillin (*24*), another caveolae-associated integral membrane protein abundantly present in neurons, cofractionated exclusively with the low-density fraction, exhibiting a sharp light-scattering opaque band (fractions 4−6 in Figure 4A). Other caveolae-associated proteins including fyn, src, and Gβ (β subunit of heterotrimeric G) were also associated with the same fraction. However, APP was undetectable in the low-density fraction prepared by this protocol using Triton X-100 for the initial step (data not shown), an observation which is consistent with the previous result (*25*). ELISA quantitation showed that Triton-insoluble Aβ was partitioned mainly into the two fractions: the low-density fraction (fractions 4−6) and the

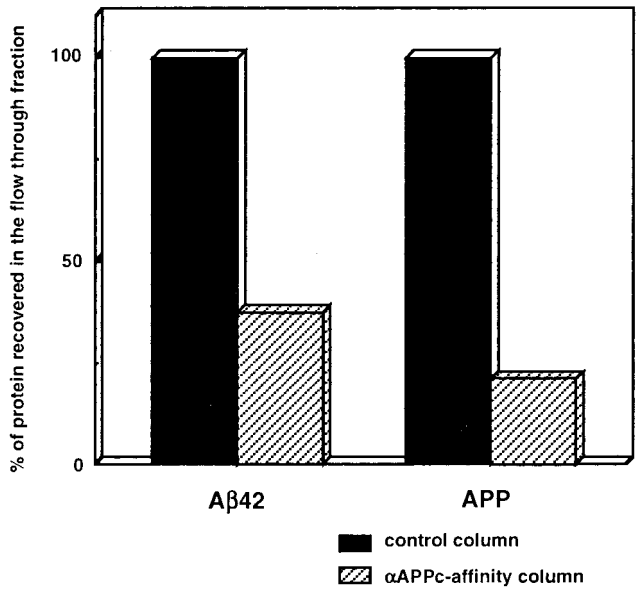

FIGURE 2: Effect of APP on the ELISA quantitation of Aβ in the Triton-soluble fraction. SH-SY5Y cells were lysed with 1% Triton X-100, and the Triton-soluble fraction was applied onto an anti-APPc immunoaffinity column or a control column conjugated with rabbit IgG. The levels of Aβ and APP in the flow-through fraction were quantitated by ELISA and Western blotting, respectively. The similar results were obtained from three independent experiments. Aβ42 or APP recovered from the control column is assumed to be 100% (closed bar). The level of Aβ42 or APP recovered from anti-APPc column is expressed as a percent relative to that from the control column (shaded bar).

Table 1: Aβ Quantitation in Triton-Soluble and Triton-Insoluble Fractions[a]

Aβ	Triton-soluble (fmol/mg)	Triton-insoluble (fmol/mg)
Aβ1-40[b]	0.342 ± 0.048	0.949 ± 0.128
Aβx-40[c]	0.251 ± 0.054	0.918 ± 0.156
Aβ1-42[b]	0.753 ± 0.096	1.081 ± 0.177
Aβx-42[c]	0.503 ± 0.080	1.599 ± 0.466

[a] Data are means ±SE, based on 30 independent experiments except for Aβx-40 and Aβx-42, in Triton-soluble fraction (15 independent experiments). [b] BAN50-based ELISA. [c] BNT77-based ELISA.

Triton-insoluble pellet (Figure 4B). Both Aβ40 and Aβ42 were found in the low-density fraction.

To exclude the possibility that the membrane-bound Aβ is rapidly redistributed in the presence of detergent, a detergent-free procedure was employed to purify the low-density membrane fraction. With this procedure, two light-scattering interfaces were observed after sucrose density gradient centrifugation, at 5%/35% (low-density fraction) and 35%/45% (high-density fraction) sucrose. Western blotting showed that only the low-density fraction contained flotillin (fractions 4 and 5 in Figure 5A), indicating that this low-density fraction contains caveolae-like membrane domains. In fact, when NIH3T3 cells were similarly fractionated, flotillin and caveolin were colocalized to these low-density fractions, and interestingly, high-density fractions were not observed. ELISA quantitation of each fraction showed that there were at least two peaks of Aβ, especially of Aβ40 (fractions 4 and 5 and fractions 8 and 9 in Figure 5B), exactly corresponding to the two opaque interfaces. More than half of the insoluble Aβ40 or Aβ42 was recovered in the low-density membrane fraction. Thus, the two different protocols

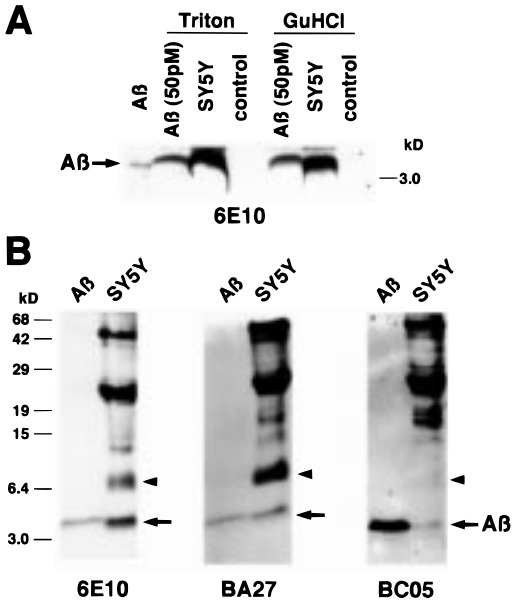

FIGURE 3: Aβ in the Triton-insoluble fraction as confirmed by immunoprecipitation and immunoaffinity chromatography. (A) Immunoprecipitation of Aβ in the Triton-soluble (Triton) or Triton-insoluble (GuHCl) fraction of SH-SY5Y cells using BAN50. The immunoprecipitates were separated on a Tris-tricine gel (16.5% T, 3% C) and analyzed by sensitive Western blotting with 6E10. Synthetic Aβ42 (50 pM) dissolved in the same solution was similarly processed as a positive control. Mouse IgG was used instead of BAN50 as a negative control. Synthetic Aβ1−42 (10 pg) was loaded in the left-most lane. (B) The Triton-insoluble fraction from SH-SY5Y cells was applied onto a 6E10-immobilized immunoaffinity column, and bound proteins were analyzed by Western blotting with 6E10, BA27, or BC05. Synthetic Aβ1−40 or Aβ1−42 (10 pg) was loaded in the left-most lane on each panel. In addition to the Aβ band at 4 kDa (indicated by arrow), an additional band at 7∼8 kDa (indicated by arrowhead) was labeled with 6E10 and BA27, and very faintly with BC05. Heavily stained bands at ∼55 and ∼25 kDa were labeled by the secondary antibody alone, thus probably representing IgG heavy and light chains, respectively. Other bands with molecular masses more than 15 kDa were also due to the cross-reactivities with the secondary antibody.

showed that relatively large pools of intracellular Aβ40 and Aβ42 are associated with low-density membrane domains.

DISCUSSION

We have shown here that (i) intracellular Aβ is present in the Triton-insoluble fraction as well as in the Triton-soluble fraction of SH-SY5Y cells; (ii) each fraction contains both Aβ40 and Aβ42; and (iii) a substantial proportion of the Triton-insoluble Aβ is associated with the low-buoyant density membrane domain. Our observation that the two-site ELISA for Aβ crossreacts with APP may require re-evaluation of the previous results obtained by ELISA. In this context, BA27 should be a powerful tool because it is a truly end-specific antibody.

More than half of the total intracellular Aβ40 and Aβ42 was recovered into the Triton-insoluble fraction (Table 1). This estimation is compatible with the data by immunoprecipitation/Western blotting (Figure 3A). Approximately one-half of the Aβ in the Triton-insoluble fraction appears to be fractionated into low-density membrane fractions accompanying flotillin and other specific resident proteins (Figure 4B). Corresponding fractions prepared by the detergent-free protocol were found to contain roughly half of the intrac-

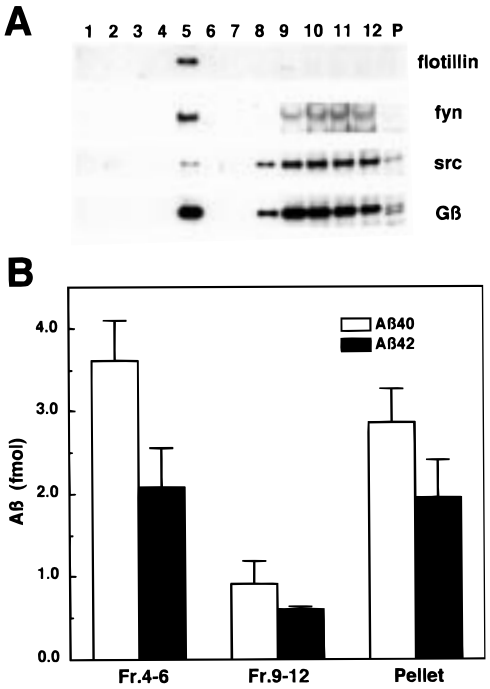

FIGURE 4: Fractionation of Triton-insoluble Aβ into detergent-insoluble, low-density membrane fractions. SH-SY5Y cells were homogenized in the presence of 1% Triton X-100 and fractionated by sucrose density gradient centrifugation as described under Experimental Procedures. (A) Fractions (each 1 mL) were collected from the top. The pellet (P) was resuspended in 1 mL of MBS by homogenization and solubilized with Laemmli sample buffer. An aliquot from each fraction was analyzed by Western blotting with anti-flotillin, anti-fyn, anti-src, and anti-Gβ antibodies. (B) After the pooled fractions were centrifuged, the resultant pellet was extracted with 6 M guanidine hydrochloride and subjected to BAN50-based ELISA for quantitation of Aβ40 (open bar) and Aβ42 (closed bar). Data are expressed as means ±SE (three independent experiments).

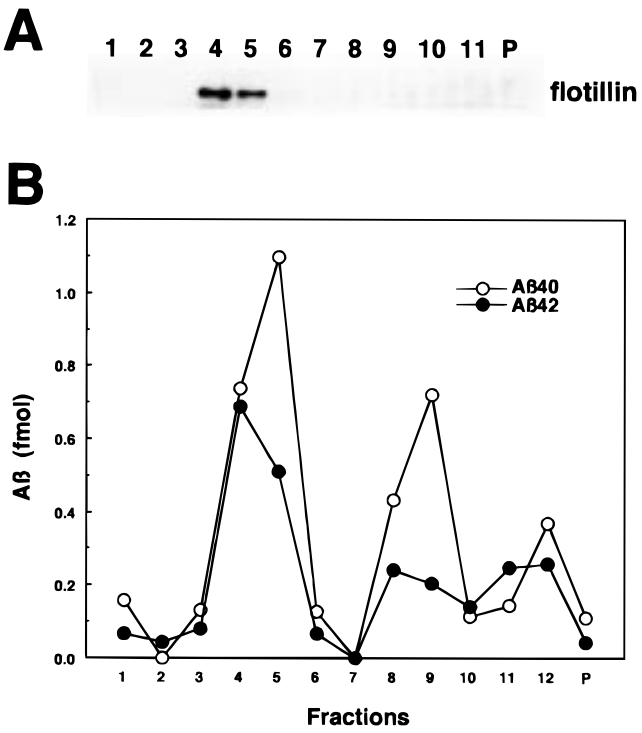

FIGURE 5: Association of Aβ with low-density membrane fractions in the absence of detergent. SH-SY5Y cells were harvested and subjected to the detergent-free purification protocol. The cells were homogenized in 0.5 M sodium carbonate, pH 11.0. After sonication, the homogenate was fractionated by relative buoyancy in a discontinuous sucrose gradient (5%/35%/45%). Fractions were collected from the top and processed as described in Figure 4. (A) An aliquot from each fraction was analyzed by Western blotting with anti-flotillin antibody. (B) Aβ40(○) or Aβ42(●) in each fraction was pelleted and quantitated by BAN50-based ELISA as described in Figure 4. Similar results were obtained from two independent experiments.

ellular, sodium carbonate-insoluble Aβ40, and presumably a similar proportion of intracellular insoluble Aβ42. These observations are consistent with the results in a very recent report on CHO cells overexpressing APP and rat brains (*26*). The association of Aβ with low-density membrane domains may be due to its association with sphingolipid and cholesterol composing an ordered phase of membrane domains. GM1 ganglioside, one of the major components of these domains, or cholesterol was shown to interact with Aβ in vitro (*13*, *27*). In particular, GM1-bound Aβ was found in diffuse plaque-enriched brains (*12*).

The Triton-insoluble intracellular Aβ appears to be stable, a finding which is consistent with the recent discovery of a large pool of insoluble, but formic acid-solubilized Aβ within NT2N cells (*28*). Thus, it is quite possible that the formic acid-solubilized Aβ pool corresponds with the Triton-insoluble, but guanidine hydrochloride-solubilized, pool of Aβ shown here, although Aβ42 is more prominent than Aβ40 in the former case. Our preliminary experiments have shown that treatment of cells with brefeldin A or monensin greatly decreased the secretion of Aβ, but had no significant effect on the levels of Triton-insoluble Aβ. This strongly suggests that Triton-insoluble Aβ is not on the route to the secretory pathway. It was reported that one of the Aβ generation sites within the cell is ER and/or Golgi compartments (*5*−*8*). However, it is still unclear whether the Aβ generated in these sites is secreted. At least, the Aβ42 generated in the ER is claimed not to be secreted from NT2N cells (*6*). Thus, a plausible explanation for the origin of Triton-insoluble Aβ is that a fraction of the Aβ generated in the ER and/or trans Golgi network becomes associated with the distinct membrane domain and is transported from the Golgi cisternae to the plasma membrane via rafts. Another possibility is that secreted Aβ is internalized into the plasma membrane (*29*) by specific interaction with the distinct membrane domain.

Besides the Aβ at 4 kDa, we found a larger fragment exhibiting Aβ immunoreactivities in the Triton-insoluble fraction. This fragment was labeled on Western blotting with BA27 and 6E10, but not with anti-APP571-580. This suggests that this band represents SDS-stable Aβ dimers, because its mobility on SDS−PAGE was indistinguishable from that of the dimers generated from synthetic Aβ (data not shown). The SDS-stable dimer was originally found in the amyloid core fractions (*30*) and extensively characterized by Roher and colleagues (*31*). This group demonstrated that the Aβ dimers do not assemble into amyloid fibrils unlike (dissociable) synthetic Aβ, but generate granular particles. A further striking characteristic of the Aβ dimer is that, in its presence, microglia kill neurons in culture. We recently found that the SDS-stable Aβ dimer at 6∼8 kDa already accumulated in the insoluble fraction from some aged human brains in which Aβ accumulation is undetectable by either immunocytochemistry or by ELISA (*11*). Thus it is possible that intracellular Triton-insoluble Aβ dimers may be the

species initially deposited in the brain. These SDS-stable Aβ dimers may be transported via rafts to the cell surface and shed into the extracellular space in a membrane-bound form under altered conditions.

Alternatively, but not mutually excluded, SDS-stable Aβ dimers in the Triton-insoluble compartment could accumulate intracellularly, leading to neuronal dysfunction. Although no evidence has thus far been reported for intracellular Aβ accumulation in subsets of neurons in AD brain, a re-evaluation should be required. This is because (i) Aβ accumulates during aging within leptomeningeal cells (*32*) and vascular smooth muscle cells (*33*) and (ii) in inclusion body myositis exhibiting unusual similarities to AD, amyloid fibrils are formed within the affected skeletal muscle fibers (*34*).

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