

Receptor-associated Protein Interacts with Amyloid- β Peptide and Promotes Its Cellular Uptake^{*S}

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Takahisa Kanekiyo[†] and Guojun Bu^{†S1}

From the Departments of [†]Pediatrics and ^SCell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¹Institute for Biomedical Research, Xiamen University, Xiamen 361005, China

Brain amyloid- β (A β) peptide accumulation and aggregation are critical events in the pathogenesis of Alzheimer disease. Increasing evidence has demonstrated that LRP1 is involved in Alzheimer disease pathogenesis. The physiological ligands of LRP1, including apoE, play significant roles in the cellular clearance of A β . The receptor-associated protein (RAP) is a specialized chaperone for members of the low density lipoprotein receptor family. RAP shares structural and receptor-binding properties with apoE. Here, we show that RAP binds to both A β 40 and A β 42 in a concentration-dependent manner and forms complexes with them. Fluorescence-activated cell sorter analysis showed that RAP significantly enhances the cellular internalization of A β in different cell types, including brain vascular smooth muscle, neuroblastoma, glioblastoma, and Chinese hamster ovary cells. This effect of RAP was confirmed by fluorescence microscopy and enzyme-linked immunosorbent assay. RAP binds to both LRP1 and heparin; however, the ability of RAP to enhance A β cellular uptake was blocked by heparin and heparinase treatment but not by LRP1 deficiency. Furthermore, the effects of RAP were significantly decreased in heparan sulfate proteoglycan-deficient Chinese hamster ovary cells. Our findings reveal that RAP is a novel A β -binding protein that promotes cellular internalization of A β .

Increasing evidence demonstrates that members of the low density lipoprotein receptor (LDLR)² family play important roles in the pathogenesis of Alzheimer disease (AD). These receptors play roles in the metabolism of the amyloid precursor protein (APP) and amyloid- β (A β) peptides (1–3). The accu-

mulation and aggregation of A β cleaved from APP in the brain are thought to be the critical steps in the pathogenesis of AD (1–3). In particular, LRP1 (LDLR-related protein 1) facilitates APP endocytic trafficking and processing to A β (4–6). LRP1 also mediates the metabolism of A β in neurons (7), glial cells (8, 9), and brain vessels (10–12). The physiological ligands of LRP1, in particular apoE and α_2 -macroglobulin, function as A β chaperones and facilitate A β clearance (1, 13).

The 39-kDa receptor-associated protein (RAP) is a specialized chaperone for members of the LDLR family, including LRP1 (14, 15). Whereas most other endoplasmic reticulum (ER) chaperones function primarily in substrate folding, RAP functions in both receptor folding (16) and subsequent trafficking (14) by blocking premature ligand binding during receptor maturation. RAP binds to LRP1 at multiple sites (14, 15) with high affinity ($K_D = 1$ –10 nM) (17). It also has been used extensively as an antagonist for LRP1. RAP was initially discovered as a protein that co-purifies with LRP1 from human placenta (18, 19). Structurally, human RAP is composed of 323 amino acids and has three independent domains (14) connected by flexible linkers (20). The C-terminal domain (domain 3) of RAP mediates folding and trafficking of receptors, whereas the N-terminal domains (domains 1 and 2) control the binding of certain ligands to receptors (21). The N-terminal domains of RAP and apoE both contain a four-helix bundle that includes a receptor-binding site, whereas their C-terminal domains are flexible (22). Additionally, both RAP and apoE contain clusters of basic amino acid residues that mediate binding to both receptors and heparin (23–26). Thus, RAP shares structural and receptor-binding properties with apoE.

In our studies of the cellular metabolism of A β , we noticed that RAP facilitates A β uptake. We hypothesized that RAP is a novel A β -binding protein. In this study, we tested potential interaction A β and RAP *in vitro*. We also analyzed the effect of exogenous RAP on A β cellular uptake using human brain vascular smooth muscle cells (HBVSMC), mouse neuroblastoma N2a cells, human glioblastoma U87 cells, and human neuroblastoma SH-SY5Y cells because these cells have been demonstrated to play roles in A β cellular uptake (7, 8, 10). We found that RAP interacts with A β and promotes its cellular uptake and that cell-surface heparan sulfate proteoglycan (HSPG) might mediate this function.

EXPERIMENTAL PROCEDURES

Materials—A β 40, A β 42, and 5(6)-carboxyfluorescein (FAM)-labeled A β 40 were purchased from AnaSpec (San Jose, CA). A β peptides were dissolved in dimethyl sulfoxide at 200

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¹ To whom correspondence should be addressed: Dept. of Pediatrics, Washington University School of Medicine, Campus Box 8208, 660 South Euclid Ave., St. Louis, MO 63110. Fax: 314-286-2894; E-mail: bu@wustl.edu.

² The abbreviations used are: LDLR, low density lipoprotein receptor; AD, Alzheimer disease; APP, amyloid precursor protein; A β , amyloid- β ; RAP, receptor-associated protein; ER, endoplasmic reticulum; HBVSMC, human brain vascular smooth muscle cells; HSPG, heparin sulfate proteoglycan; FAM, 5(6)-carboxyfluorescein; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; MEF, mouse embryonic fibroblast; DMEM, Dulbecco's modified essential medium; CHO, Chinese hamster ovary; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; HS, heparan sulfate.

μM and kept in $-80\text{ }^\circ\text{C}$ before use. Recombinant RAP and Mesd (mesoderm development) were produced and purified as described previously (27, 28).

Gel Filtration Chromatography—A β 40 (500 nm) was incubated in phosphate-buffered saline (PBS) with or without 2.5 μM RAP for 2 h at 37 $^\circ\text{C}$. Samples (500 μl) were injected onto a Superdex 75 10/30 HR column (GE Healthcare) attached to a fast protein liquid chromatography system with 50 mM ammonium acetate (pH 8.5) as the elution buffer. Fractions of 500 μl each were collected at a flow rate of 0.5 ml/min, and the concentration of A β within each fraction was determined by sandwich enzyme-linked immunosorbent assay (ELISA) using anti-A β 40 antibody 2G3 for capturing and biotin-conjugated antibody 3D6 for detection (29). Additionally, some fractions were analyzed by 12.5% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride-nitrocellulose membrane (Immobilon-P) for Western blot analysis using anti-A β antibody 6E10 or anti-RAP antibody.

Detection of the A β -RAP Complex—A β (1 μM) was incubated with or without RAP (100–1000 nM) for 1 h at 37 $^\circ\text{C}$ in PBS. For cross-linking of A β with RAP, 4 μl of glutaraldehyde (0.625% diluted from a 25% stock) was added to samples (40 μl), followed by the addition of 11 μl of NaBH₄ (6.6 mg/ml in 0.1 M NaOH) to stabilize the Schiff base formed between the aldehyde and amines. After incubation for 10 min at room temperature, 20 μl of SDS-PAGE sample buffer containing 10% 2-mercaptoethanol and 20% sucrose was added (30). Samples were separated by 12.5% SDS-PAGE and Western-blotted using anti-A β antibody 6E10 or anti-RAP antibody.

Cell Culture—HBVSMC were purchased from ScienCell (Carlsbad, CA) and cultured under standard culture conditions according to the manufacturer's protocol. Wild-type mouse embryonic fibroblast (MEF) cells (MEF1), LRP1-deficient MEF cells (MEF2), human glioblastoma U87 cells, and human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum and maintained at 37 $^\circ\text{C}$ in humidified air containing 5% CO₂, as described previously (31–34). Mouse neuroblastoma N2a cells were cultured in DMEM and Opti-MEM I (1:1) containing 5% fetal bovine serum. All Chinese hamster ovary (CHO) cell lines were grown in Ham's F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate (35).

Detection of Cell-associated A β by ELISA—Cells were plated onto 24-well dishes and allowed to grow to 90% confluency. Cells were incubated with A β 40 or A β 42 (100 nM) for 1 h at 37 $^\circ\text{C}$ in serum-free DMEM. After washing three times with PBS, cells were dissolved in 5 M guanidine in 50 mM Tris-HCl (pH 8.0). Samples were diluted 10-fold in DMEM and analyzed by sandwich ELISA for human A β 40 (antibody 2G3) and human A β 42 (antibody 21F12), both detected with biotin-conjugated antibody 3D6.

A β Cellular Internalization—Cells were cultured on the glass bottom culture dishes (MatTek Corp., Ashland, MA) at 37 $^\circ\text{C}$ for at least 24 h before experiments. After incubation with FAM-labeled A β 40 (500 nm) at 37 $^\circ\text{C}$ for 4 h in serum-free DMEM in the presence or absence of RAP (500 nM), the fluorescence of A β 40 was observed by confocal laser scanning flu-

orescence microscopy (Model LSM 510 inverted microscope, Carl Zeiss, Jena, Germany) at 488-nm argon excitation using a 510–535-nm bandpass barrier filter.

Knockdown of LRP1 by Small Interfering RNA (siRNA)—Knockdown of LRP1 was performed as described previously (36). Single-stranded, LRP1-specific, sense and antisense RNA oligonucleotides were synthesized by Ambion (Austin, TX). Double-stranded RNA molecules were generated according to the manufacturer's instructions. Cells were transfected with siRNA (120 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications and used for analysis 48 h after transfection.

Fluorescence-activated Cell Sorter (FACS)-based A β Internalization Assay—Cells were plated onto 12-well dishes and allowed to grow to 90% confluency. Cells were incubated with FAM-labeled A β 40 (500 nm) in serum-free DMEM in the presence of nonimmune rabbit IgG or RAP (500 nM). In some experiments, various concentrations of heparin (Elkins-Sinn Inc., Cherry Hill, NJ) and heparinase I (Sigma) were used. Cells were washed three times with PBS and then removed from the plate using cell dissociation solution (Sigma). Cells were centrifuged at 1400 $\times g$ for 2 min, resuspended in 100 μl of PBS containing Pronase (0.5 mg/ml; Roche Applied Science), and incubated at 4 $^\circ\text{C}$ for 30 min. Cells were washed and resuspended in 300 μl of PBS containing 1.5% fetal bovine serum, 1% NaN₃, and 1% paraformaldehyde. Cells (1×10^4) from each sample were analyzed for fluorescence on a FACSCalibur machine (BD Biosciences). Cells without any exposure to FAM-labeled A β were used as a control for background fluorescence.

Statistical Analysis—All quantified data represent an average of triplicate samples. Error bars represent S.D. Statistical significance was determined by Student's *t* test, and $p < 0.05$ was considered significant.

RESULTS

RAP Binds to A β Peptides in Vitro—To examine whether RAP is a binding protein for A β , RAP (2.5 μM) was incubated for 2 h at 37 $^\circ\text{C}$ with A β 40 (500 nm) in PBS, and the resulting mixture was subjected to gel filtration chromatography with a Superdex 75 column run on a fast protein liquid chromatography system. After incubation with RAP, A β detected by ELISA appeared to separate into two distinct peaks, which corresponded to the void and elution volumes of free A β (Fig. 1A, peaks *a* and *b*, respectively). In contrast, when A β was incubated with buffer alone, only a single peak was detected, which corresponded to the elution volume of free A β (Fig. 1A, peak *b*). To analyze the molecular nature of the A β peaks, aliquots of peak fractions were subjected to SDS-PAGE and Western blotting. After column chromatography of the A β /RAP mixture, a band was detected by anti-A β antibody 6E10 in the void volume peak, consistent with the results from ELISA (Fig. 1B). Although the molecular size of A β in the void volume peak (peak *a*) was the same as that in the elution volume peak (peak *b*), Western blotting for RAP showed that the void volume peak indeed contained RAP. These results suggest that RAP interacts with A β in solution to form molecular complexes; however, these complexes might be separated during SDS-PAGE. To confirm the binding of RAP to A β , we incubated A β 40 or A β 42

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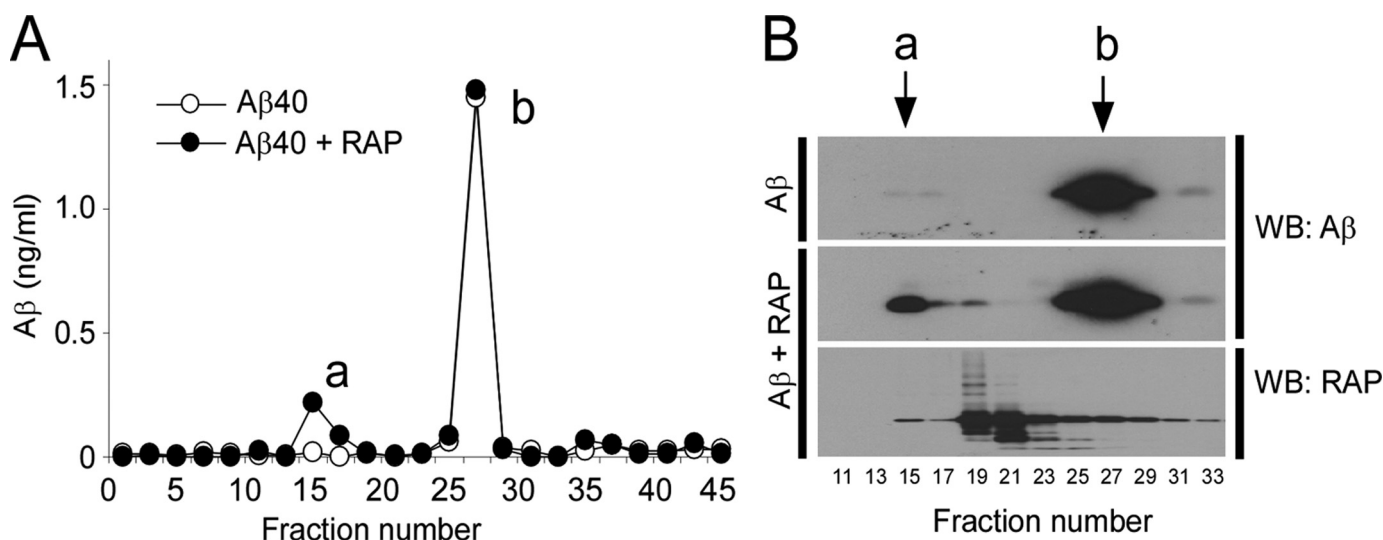


FIGURE 1. Interaction between RAP and A β . *A*, A β 40 (500 nM) was incubated with either 2.5 μ M RAP (●) or buffer alone (○) for 2 h at 37 °C and subjected to Superdex 75 gel filtration chromatography. The concentrations of A β in every other fraction were determined by ELISA. *B*, selected fractions after fast protein liquid chromatography were subjected to 12.5% SDS-PAGE and analyzed by Western blotting (WB) for A β and RAP. Arrows *a* and *b* indicate the first and second peaks from *A*, respectively.

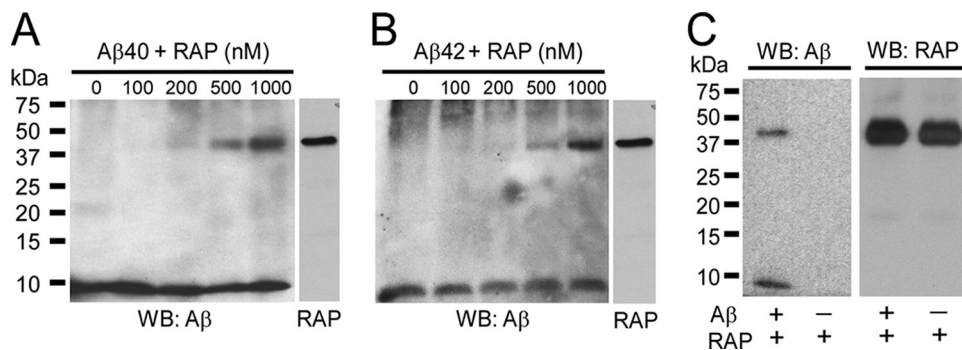


FIGURE 2. Complex formation between RAP and A β . A β 40 (1000 nM; *A*) and A β 42 (1000 nM; *B*) were incubated with increasing concentrations of RAP (50, 100, 200, 500, and 1000 nM) for 1 h at 37 °C in PBS. Samples were analyzed by 12.5% SDS-PAGE after cross-linking with glutaraldehyde, followed by Western blotting (WB) for A β . *C*, the A β -RAP complex was detected by Western blotting for A β and RAP. RAP (1 μ M) was incubated with or without 1 μ M A β 40 for 1 h at 37 °C in PBS, followed by cross-linking with glutaraldehyde, followed by Western blotting for A β (left panel). The same membrane was analyzed using anti-RAP antibody after reblotting (right panel).

(1 μ M) with various concentrations of RAP (100, 200, 500, and 1000 nM) for 1 h at 37 °C in PBS and examined the extent of A β -RAP complex formation by Western blotting after cross-linking with glutaraldehyde (Fig. 2). Western blotting for A β detected not only an A β monomer band but also a new band between the 37- and 50-kDa markers, consistent with the molecular size of the A β -RAP complex. The formation of the band depended on the RAP concentration after incubation with A β 40 (Fig. 2*A*) or A β 42 (Fig. 2*B*). Next, we confirmed the existence of A β -RAP complexes by Western blotting for A β and RAP (Fig. 2*C*). RAP (1 μ M) was incubated with or without A β 40 (1 μ M) for 1 h at 37 °C in PBS, followed by cross-linking with glutaraldehyde and Western blotting for A β . The same membrane was analyzed using anti-RAP antibody after reblotting (Fig. 2*C*, right panel). Although Western blotting for A β showed two bands in the A β /RAP mixture sample, no band was detected in the RAP-alone sample (Fig. 2*C*, left panel), indicating that anti-A β antibody does not recognize RAP. On the other hand, anti-RAP antibody recognized the upper RAP band

(37–50 kDa) but not the lower A β band (Fig. 2*C*, left panel). These results indicate that RAP forms complexes with both A β 40 and A β 42 in a concentration-dependent manner. The A β -RAP complex detected by anti-A β antibody was less abundant than the same complex detected by anti-RAP antibody. This might be due to the difference in sensitivity of these antibodies.

RAP Enhances A β Association with HBVSMC—To investigate whether RAP enhances A β cellular uptake, we used HBVSMC. A β deposition is often detected in the smooth muscle layer of the small artery as cerebral amyloid angiopathy in AD patients, and smooth muscle cells have been

demonstrated to internalize A β *in vitro* (10). A β 40 or A β 42 (100 nM) was added to HBVSMC in culture in the presence of RAP at various concentrations (50–1000 nM), and the amount of cell-associated A β was analyzed by ELISA after incubation for 1 h at 37 °C. When cells were incubated with 100 nM A β 40 for 1 h at 37 °C, 0.3% of the added A β 40 was associated with HBVSMC. After incubation of A β 40 with RAP, the association of A β with cells was increased in a concentration-dependent manner (Fig. 3*A*). Similar results were obtained for A β 42 (Fig. 3*A*). Cell-associated A β 40 and A β 42 in the presence of RAP at 500 nM were increased to 2.4- and 2.0-fold of the levels without RAP, respectively (Fig. 3*B*). These results indicate that RAP enhances the association of both A β 40 and A β 42 with HBVSMC in a concentration-dependent manner.

RAP Enhances A β Internalization by HBVSMC—To confirm the ability of RAP to promote A β internalization, HBVSMC were cultured with fluorescently labeled A β 40 and analyzed by confocal laser scanning microscopy. When cells were incubated

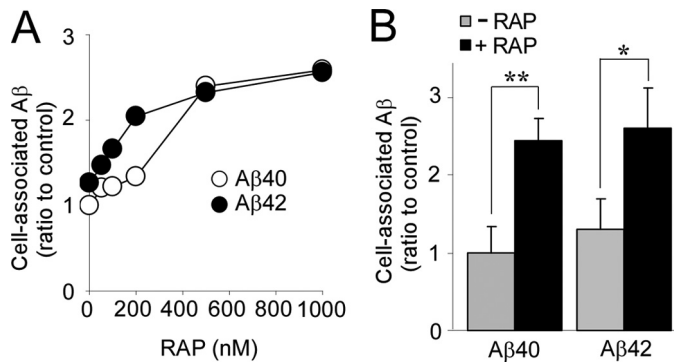


FIGURE 3. Increase in cell-associated A β by RAP in brain vascular smooth muscle cells. *A*, effect of RAP on the cellular association of A β in HBVSMC. Cells were incubated with A β 40 (100 nM) or A β 42 (100 nM) in the absence or presence of increasing concentrations of RAP (50, 100, 200, 500, and 1000 nM) for 1 h at 37 °C in serum-free DMEM. The amounts of cell-associated A β 40 (○) and A β 42 (●) were measured by ELISA. *B*, amounts of cell-associated A β 40 and A β 42 in HBVSMC measured by ELISA. Cells were incubated with A β 40 (100 nM) or A β 42 (100 nM) in the absence (gray bars) or presence (black bars) of 500 nM RAP. Error bars represent the mean \pm S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ (significantly different from the amount of cell-associated A β without RAP).

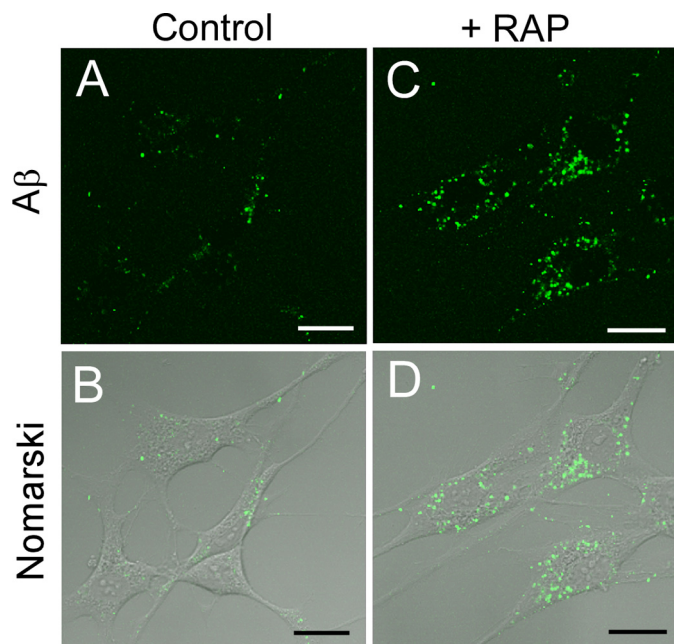


FIGURE 4. Enhancement of A β cellular uptake by RAP in brain vascular smooth muscle cells. The effect of RAP on the cellular uptake of A β in HBVSMC was analyzed using confocal laser scanning microscopy. HBVSMC were treated with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in serum-free DMEM. A β was detected in the intracellular space in a punctate pattern (*A* and *B*). In the presence of 500 nM RAP, the cellular uptake of A β was significantly enhanced (*C* and *D*). The upper and lower panels are FAM-labeled A β 40 and merged images with Nomarski images, respectively. Scale bars = 20 μ m.

with 500 nM FAM-labeled A β 40 for 4 h at 37 °C, A β 40 was detected in the intracellular space in a punctate pattern, likely in endosomal and/or lysosomal compartments (Fig. 4, *A* and *B*), indicating that HBVSMC can be used as an *in vitro* model to study A β internalization. In the presence of 500 nM RAP, the internalization of FAM-labeled A β 40 was significantly increased (Fig. 4, *C* and *D*). We found that RAP had no effect on the uptake of fluorescently labeled transferrin, used as a control (supplemental Fig. 1). These results indicate that RAP enhances the internalization of A β 40 in HBVSMC.

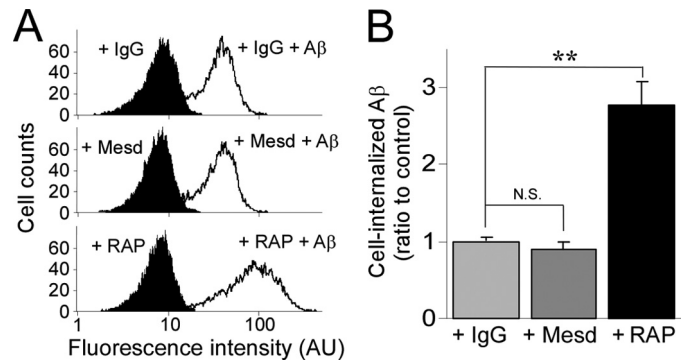


FIGURE 5. Increase in A β internalization by RAP in brain vascular smooth muscle cells. The effects of RAP on the internalization of A β in HBVSMC were analyzed by FACS. HBVSMC were incubated with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in serum-free DMEM in the presence of nonimmune rabbit IgG, Mesd, or RAP (500 nM). *A*, shown are histograms of cell fluorescence intensity after incubation with or without FAM-labeled A β 40 in the presence of IgG (upper panel), Mesd (middle panel), or RAP (lower panel). The x axis represents relative fluorescence intensity, and the y axis represents cell number. AU, arbitrary units. *B*, the change in mean cell fluorescence intensity from background fluorescence was quantified as the amount of internalized FAM-labeled A β 40. The bars indicate the amount of internalized A β in HBVSMC after incubation with IgG, Mesd, or RAP. Error bars represent the mean \pm S.D. ($n = 3$). N.S., not significant; **, $p < 0.01$ (significantly different from the amount of A β after incubation with IgG as a control).

Next, FACS was used to quantify the amount of internalized A β 40 in HBVSMC. Cells were incubated with FAM-labeled A β , treated with Pronase to remove cell-surface A β , and then analyzed for fluorescence intensity by FACS. FACS analysis revealed that the peak of fluorescence intensity of cells was shifted to the right after incubation with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in the presence of 500 nM nonimmune rabbit IgG as a control (Fig. 5*A*, upper panel), indicating that cell fluorescence intensity was increased by endocytosed FAM-labeled A β 40. Although RAP itself did not show any effect on cell fluorescence intensity, the peak of fluorescence intensity in the presence of 500 nM RAP was shifted further to the right after incubation with 500 nM FAM-labeled A β 40 (Fig. 5*A*, lower panel), indicating that RAP enhances the internalization of A β . When the changes in mean cell fluorescence intensity were quantified, cell-internalized A β 40 in the presence of RAP (500 nM) was significantly increased to 5.6-fold of that in the presence of IgG, whereas the LRP5/LRP6-specific chaperone Mesd (28) did not show any significant effect to enhance the cellular uptake of A β (Fig. 5*B*). Together, these results clearly indicate that RAP enhances the internalization of A β in HBVSMC.

RAP Enhances A β Internalization in N2a and U87 Cells—To investigate the function of RAP in A β internalization in other cell types, we used the mouse neuroblastoma cell line N2a and the human glioblastoma cell line U87. Neurons and glial cells, as well as smooth muscle cells, are known to internalize A β (7, 8). N2a cells were incubated with 500 nM FAM-labeled A β 40 for 24 h at 37 °C in the presence of 500 nM RAP or IgG as a control, and the uptake of A β was assessed by FACS. A β internalization was significantly (9.3-fold) higher in the presence of RAP compared with the control (Fig. 6*A*). In the case of U87 cells, although RAP significantly increased A β uptake to 1.4-fold of the control (Fig. 6*B*), the extent of this increase was lower compared with HBVSMC and N2a cells. These results indicate that RAP can enhance A β cellular uptake in neuronal cells, glial

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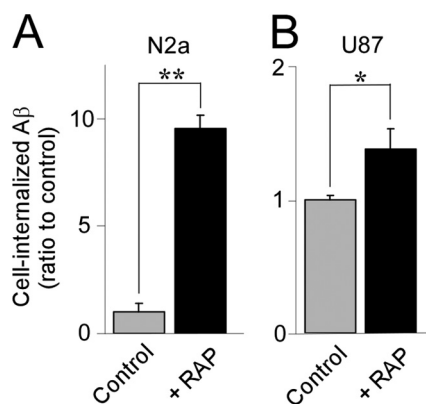


FIGURE 6. Increase in A β internalization by RAP in neuronal cells and glial cells. Mouse neuroblastoma N2a (A) and human glioblastoma U87 (B) cells were incubated with FAM-labeled A β 40 (500 nM) at 37 °C in serum-free DMEM in the presence or absence of RAP (500 nM). N2a and U87 cells were incubated for 24 and 4 h, respectively. Error bars represent the mean \pm S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ (significantly different from the amount of A β after incubation with IgG as a control).

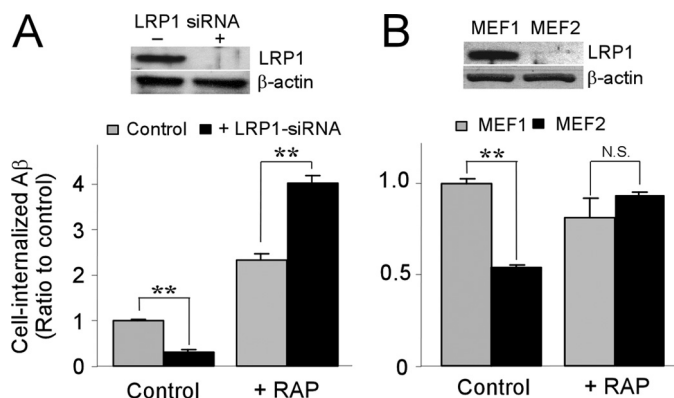


FIGURE 7. RAP promotes A β cellular uptake independently of LRP1. A, HBVSMC without (gray bars) or with (black bars) LRP1 knockdown by siRNA were incubated with FAM-labeled A β 40 (500 nM) for 4 h at 37 °C in serum-free DMEM in the presence or absence of RAP (500 nM). Western blotting (inset) showed that LRP1 expression was effectively suppressed by siRNA transfection. B, wild-type MEF1 (gray bars) and LRP1-deficient MEF2 cells (black bars) were incubated with FAM-labeled A β 40 (500 nM) for 4 h at 37 °C in serum-free DMEM in the presence of nonimmune rabbit IgG or RAP (500 nM). Western blotting (inset) showed that LRP1 was completely deficient in MEF2 cells. Error bars represent the mean \pm S.D. ($n = 3$). N.S., not significant; **, $p < 0.01$ (significantly different from the amount of A β after incubation with IgG as a control).

cells, and smooth muscle cells; however, the ability of RAP to promote A β uptake varies depending on the cell type.

RAP Mediates the Cellular Uptake of A β in an LRP1-independent Manner—LRP1 is a major A β -scavenging receptor (1), and RAP has a high affinity for LRP1. Therefore, we next investigated whether LRP1 is involved in the function of RAP to enhance A β cellular uptake. To analyze the role of LRP1 in RAP-mediated A β internalization, HBVSMC were transfected with LRP1 siRNA using Lipofectamine 2000 and used for analysis 48 h after transfection. The expression of LRP1 was significantly suppressed by siRNA (Fig. 7A, inset). LRP1-suppressed HBVSMC were incubated with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in the presence of 500 nM RAP or IgG as a control, and the internalization of A β was assessed by FACS. Knockdown of LRP1 suppressed A β internalization to 30% of that in HBVSMC treated with the vehicle Lipofectamine 2000 (Fig.

7A). However, RAP further enhanced A β internalization in LRP1-deficient HBVSMC compared with HBVSMC treated with Lipofectamine 2000 (Fig. 7A). These results indicate that RAP increases A β cellular uptake in an LRP1-independent manner.

To further examine the role of LRP1 in RAP-promoted A β uptake, we next used wild-type MEF1 and LRP1-deficient MEF2 cells (37, 38). MEF1 or MEF2 cells were incubated with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in the presence of 500 nM RAP or IgG as a control, and the uptake of A β was assessed by FACS. A β internalization in MEF2 cells was decreased to 54% of that in MEF1 cells (Fig. 7B). Although RAP showed no enhancement of A β internalization in MEF1 cells, A β internalization in MEF2 cells was significantly (1.7-fold) higher in the presence of RAP compared with the control (Fig. 7B). These results further confirm that RAP increases A β cellular uptake in an LRP1-independent manner.

HSPG Is Involved in the RAP-mediated Cellular Uptake of A β —RAP has a high affinity for heparin (24). Therefore, we next investigated whether HSPG is involved in the function of RAP to enhance A β cellular uptake. To analyze the role of HSPG in RAP-mediated A β internalization, HBVSMC and SH-SY5Y cells were incubated with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in the presence of 500 nM RAP with or without heparin, and the internalization of A β was assessed by FACS. Heparin (15 units/ml = 100 μ g/ml) did not interfere with the interaction between RAP and A β 40 (Fig. 8A). Although RAP significantly increased A β internalization in both HBVSMC (Fig. 8B) and SH-SY5Y cells (Fig. 8C), heparin suppressed the function of RAP in a concentration-dependent manner (Fig. 8D). In addition, heparinase treatment also significantly decreased the function of RAP (Fig. 8E). These results suggest that RAP increases A β cellular uptake in an HSPG-dependent manner.

To further examine the role of HSPG in RAP-promoted A β uptake, we next used wild-type CHO-K1 cells, CHO-M1 cells deficient in *N*-acetylglucosaminyltransferase/glucuronyltransferase (required for heparan sulfate (HS) biosynthesis) (39), and CHO-745 cells deficient in xylosyltransferase (required for both HS and chondroitin sulfate biosynthesis). CHO cells (40) were incubated with 500 nM FAM-labeled A β 40 for 4 h at 37 °C in the presence of 500 nM RAP, and the uptake of A β was assessed by FACS. A β internalization was significantly (2.4-fold) higher in the presence of RAP compared with the control in CHO-K1 cells (Fig. 9). However, the function of RAP in A β internalization in CHO-M1 and CHO-745 cells was suppressed to 23 and 13% of that in CHO-K1 cells, respectively (Fig. 9A). Heparin suppressed the function of RAP in CHO-K1 cells in a concentration-dependent manner but showed no effect in CHO-M1 cells (Fig. 9B). These results strongly suggest that proteoglycan may mediate the function of RAP to increase A β cellular uptake. The difference between CHO-M1 and CHO-745 cells was small compared with that between CHO-K1 and CHO-M1 cells, indicating that HSPG may be the predominant cell-surface proteoglycan that mediates RAP function.

DISCUSSION

AD is a progressive neurodegenerative disorder in which the aggregation and deposition of A β are central for its pathogen-

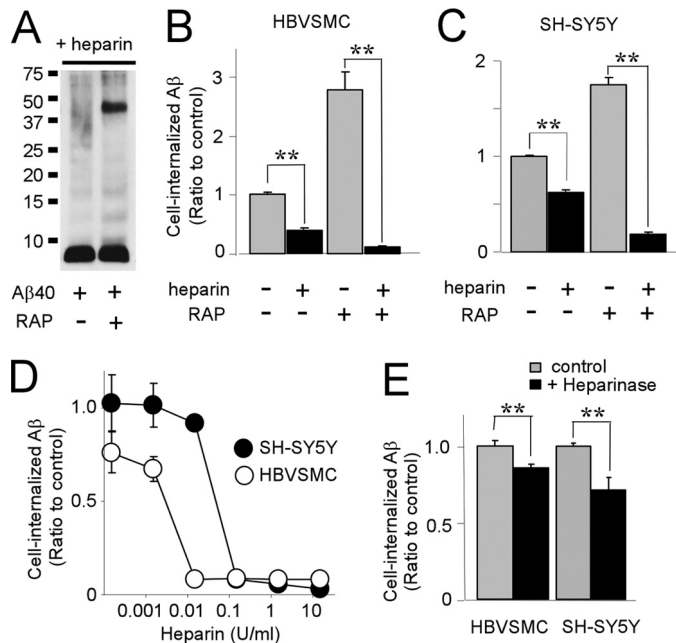


FIGURE 8. Heparin inhibits RAP function to promote A β cellular uptake. *A*, A β 40 (1 μ M) was incubated with or without RAP (1 μ M) in the presence of heparin (15 units/ml = 100 μ g/ml) for 1 h at 37 °C in PBS and analyzed by 12.5% SDS-PAGE after cross-linking with glutaraldehyde, followed by Western blotting for A β . HBVSMC (*B*) and SH-SY5Y cells (*C*) were incubated with FAM-labeled A β 40 (500 nm) with or without RAP (500 nm) for 4 h at 37 °C in serum-free DMEM in the presence or absence of heparin (15 units/ml). **, $p < 0.01$ (significantly different from the amount of A β after incubation with IgG as a control). *D*, various concentrations of heparin (0.15 milliunits/ml to 15 units/ml) were used in HBVSMC (○) and SH-SY5Y cells (●) for incubation with FAM-labeled A β 40 (500 nm) plus RAP (500 nm). *E*, cells were incubated with heparinase I (5 units/ml) for 2 h at 37 °C in serum-free DMEM, and internalization of A β was analyzed in the presence of RAP. Error bars represent the mean \pm S.D. ($n = 3$). **, $p < 0.01$ (significantly different from the amount of RAP-mediated A β internalization without heparinase treatment as a control).

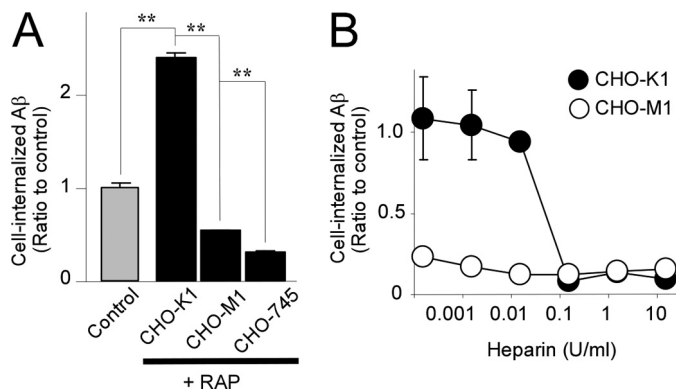


FIGURE 9. RAP promotes A β cellular uptake through HSPG. *A*, CHO-K1 (wild-type), CHO-M1 (HS-deficient), and CHO-745 (HS- and chondroitin sulfate-deficient) cells were incubated with FAM-labeled A β 40 (500 nm) for 4 h at 37 °C in serum-free DMEM in the presence RAP (500 nm). **, $p < 0.01$ (significantly different from the amount of internalized A β in CHO-K1 cells after incubation with IgG as a control). *B*, various concentrations of heparin (0.15 milliunits/ml to 15 units/ml) were used in CHO-K1 (●) and CHO-M1 (○) cells for incubation with FAM-labeled A β 40 (500 nm) plus RAP (500 nm). Error bars represent the mean \pm S.D. ($n = 3$).

esis (1–3). A β is cleaved from APP in the ER, *trans*-Golgi network, plasma membrane, and endosome and then secreted to the extracellular space (41–43). It has been suggested that the cell quality control mechanisms by chaperones prevent intracellular aggregation and deposition of A β in the healthy brain.

Although several cytosolic chaperones (44, 45) bind to A β *in vitro*, they are predicted to rarely interact with A β under physiological conditions. In this study, we revealed that an ER chaperone, RAP, binds to both A β 40 and A β 42 and forms complexes with them. Under physiological conditions, RAP functions as a chaperone within the early compartments of the secretory pathway. Immunoelectron analysis revealed that RAP localizes mainly within the ER (70%) and early Golgi compartments (24%) and less frequently on the cell surface or within compartments of the endocytic pathway (14, 31). Because RAP possesses an ER retention signal sequence (HNEL), it is efficiently retrieved from the Golgi back to the ER (14, 46). However, overexpression of RAP has been shown to induce the saturation of the retrieval system, resulting in the secretion of RAP into the extracellular space (47). Thus, it may be possible that RAP could be secreted when its expression is up-regulated under certain physiological or pathological conditions such as AD, suggesting that RAP might encounter A β in the extracellular space. Pandey *et al.* (48) showed that a polymorphism of the *LRPAP1* gene encoding RAP protein is involved in the susceptibility to degenerative dementias. The frequency of the DD genotype and D allele of the *LRPAP1* gene was significantly higher in degenerative dementias than in controls. The odds ratios for the risk of degenerative dementia were estimated to 3.76 for the DD genotype and 2.80 for the D allele (48). Additional studies are necessary to confirm this result; however, it suggests that RAP likely plays some roles in AD pathogenesis.

RAP might also play roles in APP folding, trafficking, and/or processing (49). The ability of RAP to bind A β suggests that RAP might also bind to APP and regulate its trafficking and processing. Additionally, RAP regulates the maturation and trafficking of LRP1, facilitating APP endocytic trafficking and processing to A β (50). Therefore, a RAP dysfunction may lead to a disturbance in APP and A β metabolism, resulting in A β deposition. Consistent with this hypothesis, when RAP-deficient mice were crossed with human APP transgenic mice, the amount of A β deposition was increased (51), suggesting a central role for RAP in A β metabolism and AD pathogenesis. Recently, Xu *et al.* (52) revealed that partial reductions in RAP enhance A β deposition in the APP^{swe}/PS1^{dE9} model of AD. Because partial RAP deficiency did not significantly affect the levels of LRP1 and SorLA/LR11, RAP may directly regulate APP and/or A β metabolism *in vivo*.

In addition to the ability of RAP to bind A β , we demonstrated that exogenous RAP enhances the cellular uptake of A β in different cell types, including HBVSMC, neuroblastoma cells, glioblastoma cells, and CHO cells. RAP is widely used to antagonize ligand interactions with members of the LDLR family (15). In several studies, RAP was used to antagonize LRP1-mediated A β uptake (53, 54). Although these studies focused on endothelial cells and used low concentrations of A β , their results are opposite from ours. They showed that RAP prevents A β uptake and clearance *in vitro* and *in vivo*. This discrepancy may be explained as follows. 1) The ability of RAP to regulate A β cellular uptake may be different depending on the cell type; 2) the effect of RAP may be different depending on the A β concentration; and 3) RAP may block LRP1-mediated A β uptake when the A β -LRP1 ligand complex is produced. Our

RAP Binds to A β and Enhances Its Uptake

results strongly indicate that RAP has to be used very cautiously as an antagonist of LRP1 when the cellular uptake of A β is investigated. Vascular smooth muscle cells, neurons, and glial cells can internalize A β (7, 8, 10), which is predicted to be mainly degraded. However, when the levels of internalized A β exceed the cellular capacity to degrade internalized A β , it may accumulate and deposit intracellularly, resulting in cell death. Therefore, we analyzed the cell toxicity of enhancement of A β uptake by RAP using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. We found no significant differences in cell viability in the presence or absence of RAP (supplemental Fig. 2), suggesting that RAP enhances the cellular clearance of A β without significant effects on cell viability under these experimental conditions. The accumulation of A β in the extracellular space is thought to trigger its aggregation and deposition, which contribute to AD and cerebral amyloid angiopathy. Therefore, the function of RAP to enhance the cellular clearance of A β might be protective against the pathogenesis of AD and cerebral amyloid angiopathy.

Unexpectedly, RAP enhances A β cellular uptake in an LRP1-independent manner. In LRP1-suppressed HBVSMC and LRP1-deficient MEF cells (MEF2), the effect of RAP was strongly detected. Moreover, RAP increased the internalization of A β in N2a cells, which express little LRP1 or LDLR (55). These findings suggest that the role of RAP in facilitating A β uptake is independent of the LDLR family members, despite the high affinities of RAP for them. Previous studies have demonstrated that RAP is a heparin-binding protein (24, 56). Three basic amino acid clusters (R²⁸²VSRSEK²⁸⁹, R²⁰³LRR²⁰⁶, and R³¹⁴ISRAR³¹⁹) within domain 3 of RAP contribute to high affinity binding to both RAP and LRP1 (24). HSPG is ubiquitously expressed on the cell surface in almost all mammalian cells (57). Therefore, we focused on RAP interaction with HSPG and demonstrated that HSPG is involved in the ability of RAP to promote A β cellular uptake. We found that the ability of RAP to promote A β uptake varies depending on the cell type. Analysis of HS from different mammalian tissues revealed that HS biosynthesis differs depending upon the tissue (58). The cell type discrepancies in RAP-mediated A β uptake may be due to the differences in the cell-specific HS structure. In addition, it is well known that interactions with the cell-surface HSPGs glypican and syndecan are required for the internalization of several cationic peptides (59). Glypican is glycosylphosphatidylinositol-anchored, and syndecan has a transmembrane domain (59). Among them, GPC1 (glypican-1) and SDC3 (syndecan-3) are major HSPGs in the adult brain (60, 61); therefore, we investigated the association of these HSPGs in RAP function. GPC1 and SDC3 in glial cells have been shown to be associated with A β deposits in AD (62). Although knockdown of GPC1 and SDC3 decreased the internalization of A β in HBVSMC in the absence of RAP, no inhibitory effect on RAP-mediated A β uptake was detected (supplemental Fig. 3). These results suggest that multiple HSPGs mediate the function of RAP. When one HSPG is suppressed, other HSPGs may compensate. The other possibility is that other HSPGs on the cell surface or HSPGs in the extracellular matrix (e.g. perlecan, agrin, and collagen XVIII) may be involved in RAP function. Further

studies are needed to characterize HSPGs that mediate the RAP function.

In this study, we demonstrated that RAP binds to A β and enhances its cellular uptake. Because the structure of each domain of RAP has already been determined (20, 63, 64), it may be possible to develop novel RAP-mimicking drugs to increase the cellular uptake of A β for AD therapy. Our findings thus provide a new insight into the molecular mechanism of AD pathogenesis and a potential therapeutic strategy for AD.

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