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Interaction of N-Terminal Acetyltransferase with the Cytoplasmic Domain of β-Amyloid Precursor Protein and Its Effect on Aβ Secretion

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The processing of β-amyloid precursor protein (APP) generates the amyloid β-protein (Aβ) and contributes to the development of Alzheimer's disease (AD). Elucidating the regulation of APP processing will, therefore, contribute to the understanding of AD. Many APP-binding proteins, such as FE65, X11s, and JNK-interacting proteins (JIPs), bind the motif 681-GYENPTY-687 within the cytoplasmic domain of APP. Here we found that the human homologue of yeast amino-terminal acetyltransferase ARD1 (hARD1) interacts with a novel motif, 658-HGVVEVD-664, in the cytoplasmic domain of APP695. hARD1 expressed its acetyltransferase activity in association with a human subunit homologous to another yeast amino-acetyltransferase, hNAT1. Co-expression of hARD1 and hNAT1 in cells suppressed Aβ40 secretion and the suppression correlated with their enzyme activity. These observations suggest that the association of APP with hARD1 and hNAT1 and/or their N-acetyltransferase activity contributes to the regulation of Aβ generation.

Key words: Alzheimer's disease, APP, N-terminal acetyltransferase, amyloid β-protein.

Abbreviations: ACTH, adrenocorticotropic hormone; APP, amyloid β-protein precursor; APPcyt, the cytoplasmic domain of APP; Aβ, β-amyloid; X11L, X11-like; AD, Alzheimer's disease; AICD; intracellular fragment of APP cleaved at γ-/ε-site; APLP1, amyloid precursor-like protein 1; APLP2, amyloid precursor-like protein 2; ARD1, amino-terminal acetyltransferase; CHAPS, 3-[(3-cholamidpropyl)dimethylammonio]-1-propane-sulfonic acid; FITC, fluorescein isothiocyanate; JIP, c-Jun NH_2-terminal kinase (JNK)–interacting protein; HA, haemagglutinin-epitope tag; NAT1, arylamino *N*-acetyltransferase; RT-PCR, reverse transcriptase; TRITC, tetramethylrhodamine B isothiocyanate.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized pathologically by neuronal loss and a relatively high abundance of parenchymal plaques composed largely of the amyloid β-protein (Aβ) (*1*). It is believed that high levels of Aβ in the brain initiate a crucial series of pathological events that ultimately lead to neuron loss and dementia (*2*). Aβ is generated by endoproteolytic processing of the amyloid β-protein precursor (APP) in protein secretory and endocytic pathways (*3*).

The short cytoplasmic domain of APP is responsible for modulating the intracellular trafficking and metabolism of APP and ultimately regulates Aβ production (*4*–*7*). Moreover, it was recently reported that the intracellular fragment of APP cleaved at the γ-/ε-site, designated AICD, possesses gene transactivation activity (*8*), which suggests that the short cytoplasmic tail of APP could be involved in intracellular signal transduction.

The cytoplasmic domain of APP is composed of 47 amino acids and contains at least three functional motifs. The 653-YTSI-656 motif (human APP695 isoform numbering) has been reported to be the basolateral sorting signal of APP in epithelial MDCK cells (*9*). The 667-VTPEER-672 motif, which forms an amino-terminal helix capping box structure, contributes to the stability of the overall structure of the APP cytoplasmic domain (*10*). The 681-GYENPTY-687 motif has been characterized as an endocytotic signal of APP (*18*) analogous to the NPXY motif of the LDL receptor (*19*). Deletion or substitution of amino acid(s) in the 681-GYENPTY-687 motif impairs APP endocytosis and alters Aβ production (*20*). Cytoplasmic adaptor proteins such as FE65s (*21*, *22*), X11s (*23*–*25*), and JIPs (*14*, *26*) interact with this motif through their phosphotyrosine-interaction (PI) domain. These interactions between the cytoplasmic domain of APP and cytoplasmic proteins are thought to play an important role in the regulation of APP metabolism, including Aβ generation, although the detailed mechanisms are still being investigated.

In the present study, we found that a human homologue of the yeast amino-terminal acetyltransferase ARD1 (*27*), designated hARD1, binds the APP cytoplasmic domain with its carboxy-terminal 50 amino acids. This binding occurs through a novel motif in the APP cytoplasmic domain, 658-HGVVEVD-664. hARD1 associ-

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ates with a human homologue of another yeast N-acetyltransferase, NAT1 (*28*, *29*), designated hNAT1. In yeast, NAT1 is a regulator of ARD1 and a complex composed of ARD1 and NAT1 shows enzymatic activity (*30*). The complex composed of hARD1 and hNAT1 also expresses amino-terminal acetyltransferase activity, which results in a decrease of Aβ40 generation from APP.

EXPERIMENTAL PROCEDURES

cDNA Cloning of hARD1 and Plasmid Construction—The cDNA encoding the cytoplasmic domain of APP (APPcyt: amino acid number 645–695 of the APP695 isoform) was inserted into the pGilda vector. This pGilda-APPcyt was used as bait in the Match Maker Two-Hybrid System Clontech Lab., Inc. (Palo Alto, CA) to isolate the cDNAs of APP-binding proteins from a human fetal brain cDNA library cloned into a pB42AD vector, which contains the GAL4 transactivation domain. The plasmids were transfected sequentially into the yeast strain EGY48 [MATα, ura3, his3, trp1, LexAop(X6)-LEU], which had previously been transformed with the p8op-lacZ reporter plasmid, according to the manufacturer's protocol (catalog no. PT3040-1, Clontech Lab.).

Ultimately, 96 clones were isolated that were positive for nutrient (Leu) selection and an *in vivo* β-galactosidase plate assay. The positive clones were re-transfected into *E. coli,* the nucleotide sequences were determined, and a cDNA clone encoding the carboxyl 232 amino acids of the human ARD1 homologue (hARD1) was identified as a candidate APPcyt-binding protein. The missing 5′-region was obtained by PCR and the full-length hARD1 cDNA, consisting of 235 amino acids, was re-cloned into pcDNA3 to produce pcDNA3-hARD1, pcDNA3-hARD1-HA (tagged with HA at the carboxyl-terminal), and pcDNA3-HA-hARD1 (tagged with HA at the amino-terminal). Several constructs encoding carboxyl-terminal truncated mutants of hARD1 were made using PCR techniques and cloned into the pB42AD vector. These include pB42AD-hARD1ΔC50, pB42AD-hARD1ΔC100, and pB42AD-hARD1ΔC150 lacking the carboxyl-terminal 50, 100, and 150 amino acids, respectively. Constructs encoding amino acid substitutions in the acetyl-CoA binding domain, pcDNA3-hARD1^{R82A}-HA and pcDNA3-hARD1^{G85A}-HA (tagged with HA at carboxyl-terminal), were also produced by PCR.

cDNA Cloning of hNAT1 and Plasmid Construction—A human homologue of the yeast *NAT1* gene, *hNAT1*, was found by a thorough search of human cDNA and genome databases. We isolated hNAT1 using PCR technologies and cloned it into the pcDNA3 vector to produce pcDNA3-hNAT1-FLAG (tagged with FLAG at carboxyl-terminal). Isolation of the identical cDNA has recently been reported elsewhere (*31*).

Protein Interaction Assay in Yeast—Several constructs encoding truncated and amino acid-substituted versions of APPcyt were made using PCR techniques and cloned into pGilda vectors. These include pGilda-APPcytΔC14, pGilda-APPcytΔC28, pGilda-APPcytΔN19, pGilda-APPcytΔ658–664, pGilda-APPcytY653A, pGilda-APPcytT668A, and pGilda-APPcytT668E (see Fig. 2 for schematics). The cytoplasmic domains of APLP1 and APLP2 were also produced (*24*) and cloned into pGilda vectors to produce pGilda-APLP1cyt and pGilda-APLP2cyt. These constructs were transfected into yeast with pB42AD-hARD1 as described above. Protein interaction was tested by growth under nutrient (Leu) selection.

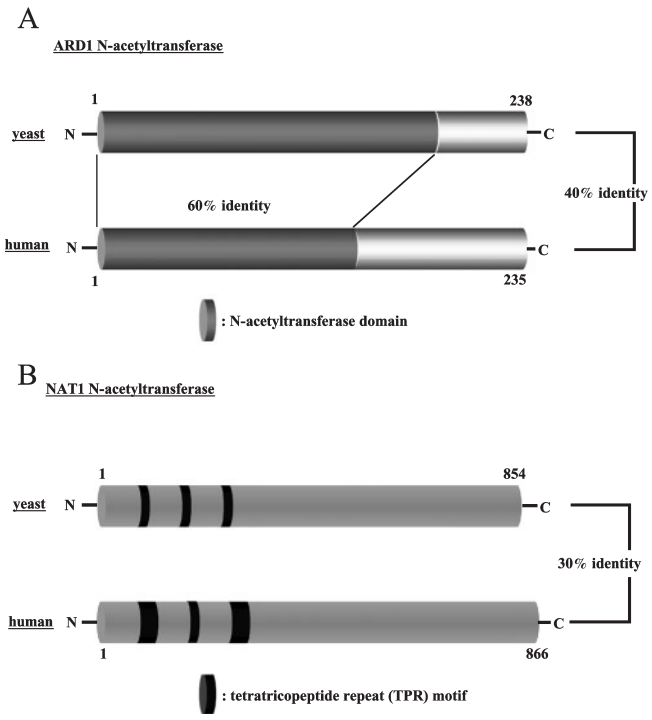

Fig. 1. **Schematic diagram of yeast and human N-terminal acetyltransferases.** Amino acid identity between the two proteins is indicated as percent homology (%). A: Comparison of yeast and human ARD1. Predicted N-terminal acetyltransferase domains showing ~60% identity are indicated by dark boxes. B: Comparison of yeast and human NAT1. Tetratricopeptide repeat motifs are indicated by dark boxes. Numbers indicate amino acid position.

Antibodies—The anti-FLAG monoclonal M2 antibody was purchased from Sigma, the anti-HA monoclonal antibody was purchased from Invitrogen, and the anti-APP cytoplasmic domain polyclonal antibody UT18 was described previously (*24*).

Co-Immunoprecipitation—Human embryonic kidney 293 (HEK293) cells were transiently transfected with the indicated combination of plasmids as described (*24*). Forty-eight hours after transfection, cells were lysed in cell lysis buffer (phosphate-buffered saline (PBS) containing 10 mM CHAPS, 5 μg/ml chymostatin, 5 μg/ml leupeptin and 5 μg/ml pepstatin A) on ice for 1 h, followed by centrifugation at 15,000 × *g* for 10 min at 4°C. The indicated antibody was added to the supernatant, and the sample was incubated on ice for 2 h. The immunocomplex was recovered with protein G-sepharose beads. The beads were washed three times with TBST buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween-20], and the proteins were eluted by boiling the beads in SDS-sample buffer [10% (v/v) glycerol, 3% (w/v) SDS, 50 mM Tris-HCl (pH 8.0), 1.2 mM EDTA, 0.005% (w/v) Bromophenol Blue, 4 M urea] containing 2-mercaptoethanol. Proteins were analyzed by Western blotting with the indicated antibodies and detected using an ECL detection kit (Amersham Pharmacia Biochemicals).

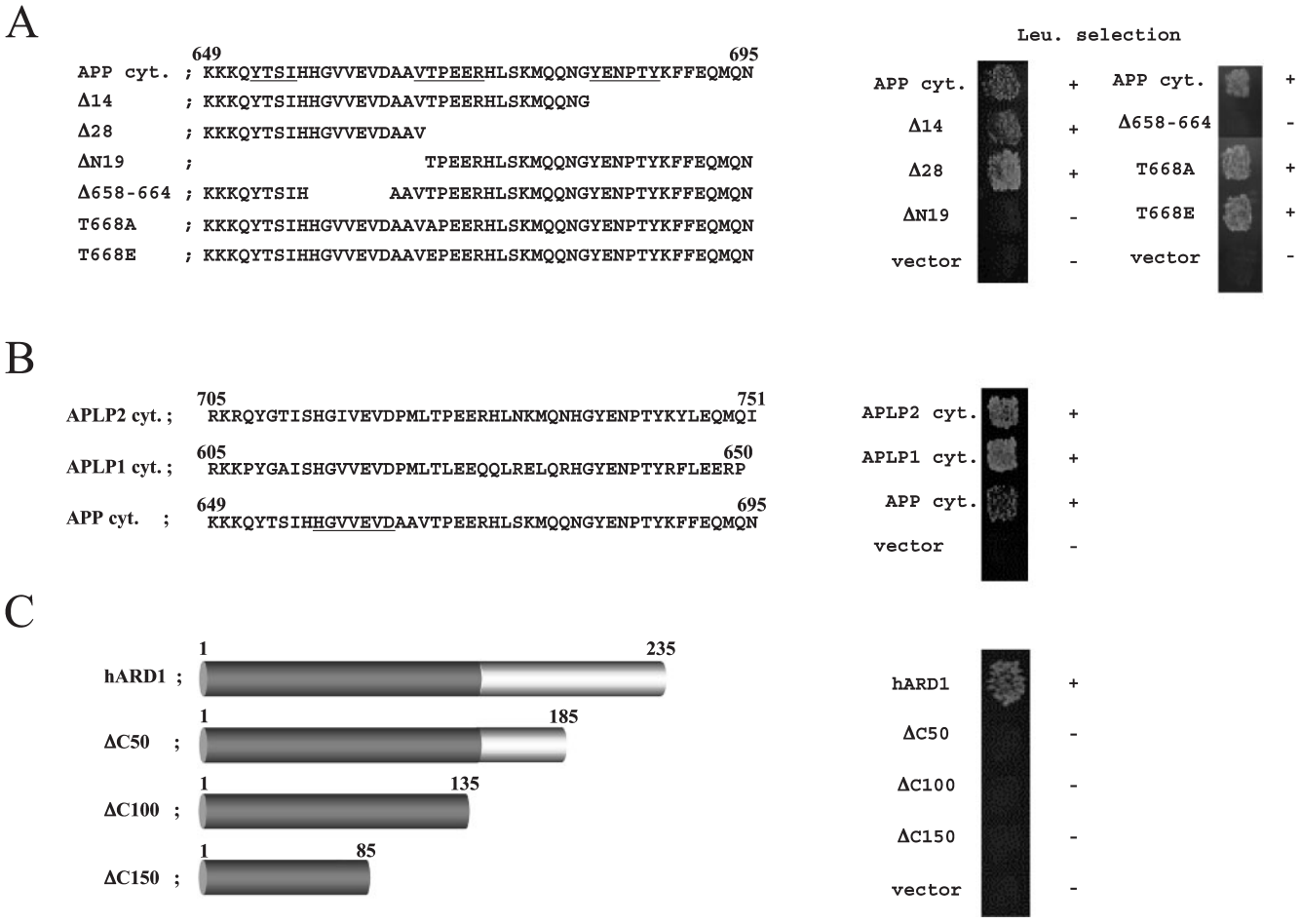

Fig. 2. **Interaction of human ARD1 with the cytoplasmic domain of APP and APP-like proteins.** The ability of hARD1 to bind the cytoplasmic domain of APP family proteins was assayed with yeast two-hybrid growth assays. Each interaction was monitored by growth on leucine-deficient plates. A: Binding ability between hARD1 and the APPcyt. The amino acid sequences of the constructs used in the assay are indicated. Numbers indicate amino acid positions in APP695. Known motifs, 653-YTSI-656, 667-VTPEER-672, and 681-GYENPTY-687, are underlined. B: Interaction of hARD1 with the cytoplasmic domain of APP family proteins. The amino acid sequences of the cytoplasmic domain of APLP2 (751 amino acid isoform), APLP1 and APP695 are shown. Underlining in APPcyt indicates the sequence that interacts with hARD1. C: Binding between the APP cytoplasmic domain and the protein constructs derived from hARD1. Each construct is shown schematically, with the predicted N-terminal acetyltransferase domains indicated by dark boxes. Numbers indicate the amino acid positions in hARD1. "Vector" indicates a study with pGilda plasmid (A and B) or pB42AD plasmid (C) only. Presence (+) and absence (–) of interactions are indicated.

Immunocytochemistry—HEK293 cells were transiently transfected with combinations of the indicated amounts of plasmids. Forty-eight hours after transfection, cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (pH 7.4) containing 4% (w/v) sucrose for 10 min at room temperature, and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min at room temperature. The cells were incubated with the indicated antibodies for 12 h at 4°C, washed well, and then incubated with FITC- and TRITC-conjugated secondary antibodies (Zymed) for 1 h at room temperature. The cells were viewed using a confocal microscope, Bio-Rrad MRC 600.

Quantification of β-Amyloid—Mouse neuroblastoma Neuro-2a (N2a) cells were transiently transfected with combinations of the indicated amounts of plasmids. The cells were supplied with fresh growth media 5 h after the start of transfection, and medium conditioned by the cells (~2 × 10^6 cells) was collected 48 h after the medium change. Aβ40 and Aβ42 were quantified with sandwich ELISA as described (*32*).

N-Acetyltransferase Enzyme Assay—The procedure for the yeast enzyme assay (*28*) was modified for use with a human enzyme. Briefly, cell lysates or their immunoprecipitates were incubated in 50 μl of reaction buffer [50 mM HEPES (pH 7.5), 150 mM KCl, 1 mM dithiothreitol] for 30 min at 37°C with 50 mM human ACTH peptide (amino acid number 1–24, Peptide Institute INC, Osaka Japan) and 25 μM [^{3}H]-acetyl CoA (0.01 μCi/μl, Amersham Pharmacia). The reaction was terminated by the addition of 8.5 μl of 0.5 M acetic acid. The radioactivity incorporated into the ACTH peptide was analyzed with a scintillation cunter.

Uptake Assay—APP uptake assay was performed as described (*20*). Briefly, triplicate culture of HEK293 cells were transiently transfected with plasmid of FLAG-APP

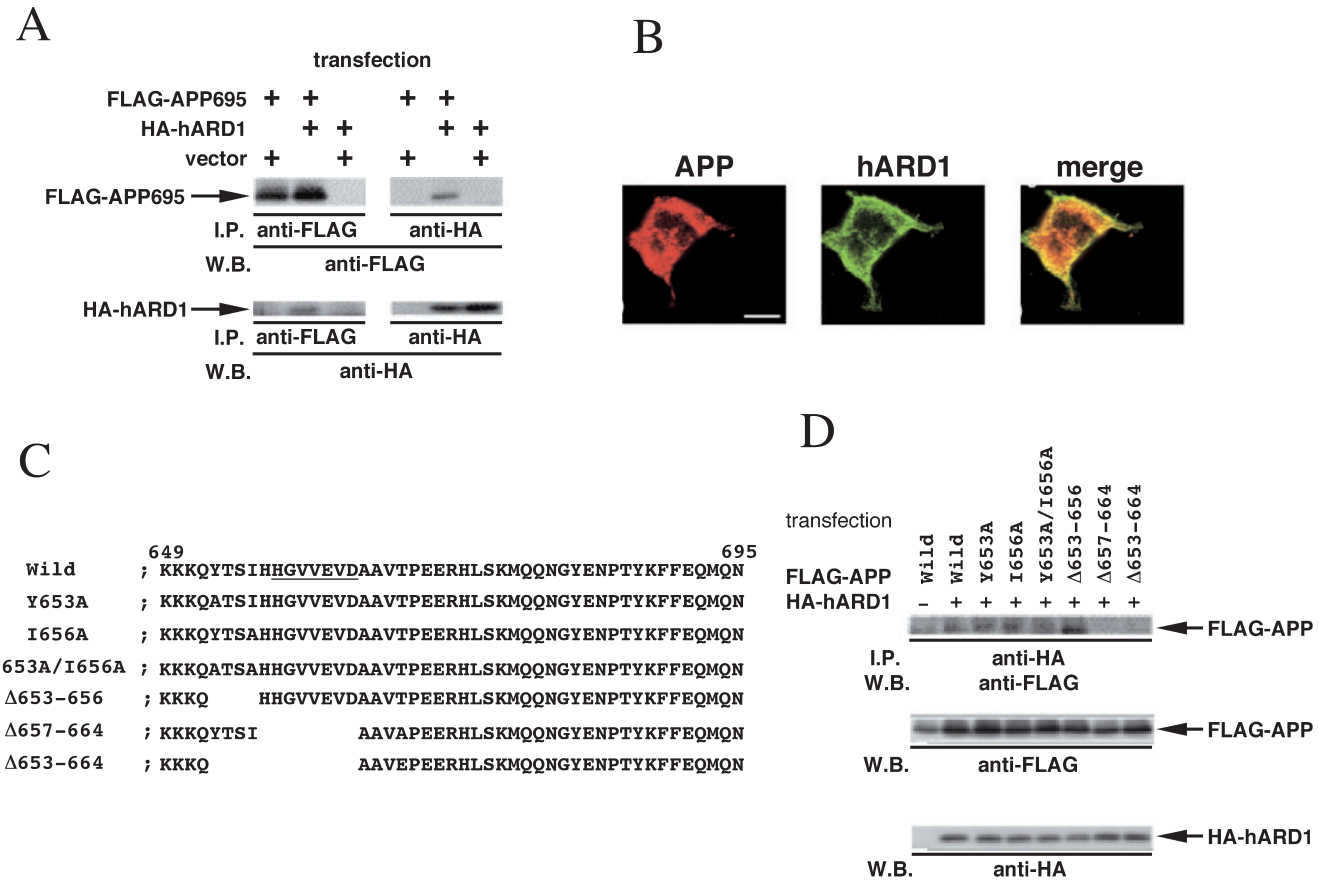

Fig. 3. **Interaction between APP and hARD1 in cells.** A: Coimmunoprecipitation of APP with hARD1. HEK293 cells were cotransfected with pcDNA3-FLAG-APP and pcDNA3-HA-hard1. To standardize the plasmid amounts, pcDNA3 vector was added. Cells were lysed and the lysate was used for immunoprecipitation (I.P.) with the indicated antibodies. The immunoprecipitates were analyzed by Western blot (W.B.) with anti-FLAG (upper panel) and anti-HA (lower panel) antibodies. B: Colocalization of APP and hARD1 in cells. HEK293 cells expressing FLAG-APP and HA-hARD1 were immunostained with anti–APP UT18 polyclonal and anti-HA monoclonal antibodies. Cells were visualized with an anti–rabbit IgG conjugated with TRITC (left panel) and an anti–mouse IgG conjugated with FITC (middle panel). The merging of the signals (right panel) indicates the colocalization of the two proteins being assessed. Scale bar, 25 μm. C: APP protein constructs used in the coimmunoprecipitation study with hARD1. The amino acid sequence of the APP cytoplasmic regions used in this study are indicated. The underline indicates the candidate sequence for interaction with hARD1 as determined by yeast two-hybrid assay (Fig. 2). Numbers indicate the amino acid position in APP695. D: HEK293 cells were transiently cotransfected with pcDNA3-FLAG-APP constructs and pcDNA3-HA-hARD1 as indicated. Forty-eight hours after transfection, cells were lysed and subjected to immunoprecipitation (I.P.) with an anti-HA antibody. The immunoprecipitates were analyzed by Western blotting (W.B.) with an anti-FLAG antibody (top panel). The middle and bottom panels indicate the expression of FLAG-APP and HA-hARD1 in cells by Western blotting of lysates.

in the presence or absence of hARD1 and hNAT1 plasmids for 72 h. To measure internalization of cell-surface protein, whole membrane surface proteins were biotinylated using sulfosuccinimidobiotin (Pierce, IL). After incubation for indicated times, cells were rapidly chilled on ice, and the biotin of cell-surface protein was removed by reduction. Then cells were lysed in cell lysis buffer on ice, followed by centrifugation at 15,000 × *g* for 10 min at 4°C. The biotinylated proteins, which were subjected to endocytosis and resistant to reduction on the cell surface, were recovered with streptavidin agarose (Pierce), and APP was detected by Western blot with anti-APP antibody UT18.

RESULTS

Isolation of cDNAs Encoding hARD1 and hNAT1—hARD1 was identified as a potential APP-binding protein in a yeast two-hybrid assay. The hARD1 cDNA consists of 235 amino acids and has ~40% identity with the amino acid sequence of the yeast ARD1 (Fig. 1A). The putative enzyme domain consisting of the amino-terminal ~160 amino acids is highly conserved (~60%), but the carboxyl-terminal regions of the two proteins are quite divergent. The acetyl-coenzyme A binding motif in the enzyme domain (see Fig. 5A) is highly conserved between human and yeast.

Because yeast ARD1 is known to interact with NAT1 (*30*), we isolated hNAT1 cDNA by RT-PCR using total RNA derived from HEK293 cells. The hNAT1 is a 95-kDa protein consisting of 866 amino acids and has ~30% iden-

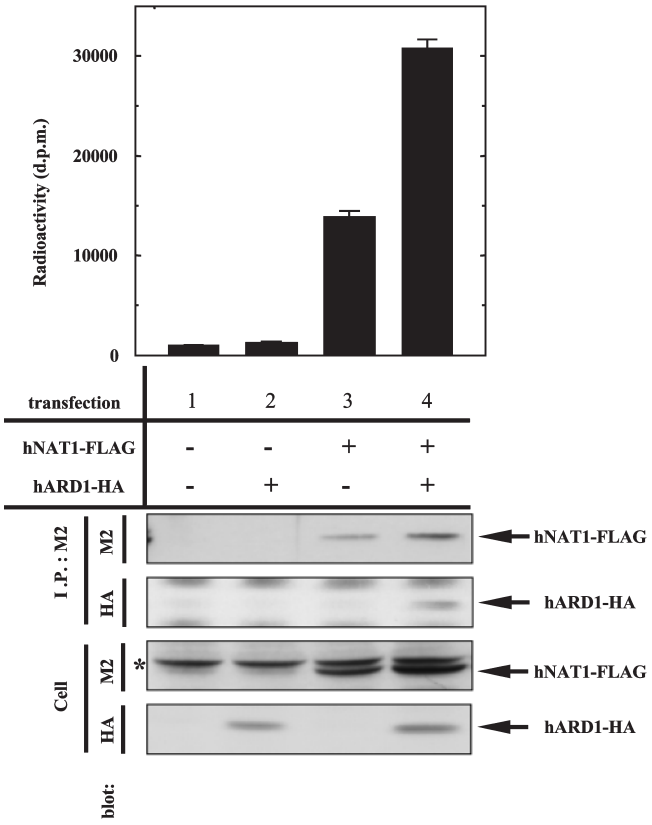

Fig. 4. **N-Acetyltransferase activity emerges with the interaction of hARD1 with hNAT1.** HEK293 cells were transfected with pcDNA3-hARD1-HA, pcDNA3-hNAT1-FLAG, or both. To standardize the plasmid amounts, pcDNA3 vector (–) was added. Using an anti–FLAG M2 antibody, the protein complex was immunoprecipitated, and assayed for enzyme activity, which is reported as radioactivities incorporated into the ACTH peptide (upper). The immunoprecipitates (I.P.) and crude cell lysates (cell) were analyzed by Western blotting with the indicated antibodies (lower). Asterisk (*) indicates a non-specific product.

tity with the amino acid sequence of yeast NAT1 (Fig. 1B). Several tetratricopeptide repeat (TPR) motifs in the amino-half are conserved. This protein was recently reported by another group (*31*).

Interaction of hARD1 with APP Family Proteins and Determination of Interaction Sites in APP and hARD1—The region of APPcyt required for the interaction with hARD1 was determined using the yeast two-hybrid system. The pB42AD-hARD1 prey vector was co-transfected with pGilda bait vector including the various APPcyt constructs (Fig. 2A). The complete sequence of APPcyt (APPcyt) and truncated constructs lacking the carboxyl-terminal 14 amino acids (Δ14) and 28 amino acids (Δ28) were able to bind hARD1, but an amino-terminal truncated construct lacking the first 19 amino acids (ΔN19) exhibited no growth on the selective agar plates. In a separate experiment, we found that the Δ658–664 construct lacking the internal 658-HGVVEVD-664 sequence lost its ability to interact with hARD1, while the T668A and T668E constructs, formed by substitution of Ala and Glu for Thr668 (*5*), retained their ability to bind to hARD1.

APP is a member of the gene family that includes APLP1 and APLP2 (*33*, *34*). The amino acid sequences of these proteins, including the 658-HGVVEVD-664 and 681-GYENPTY-687 motifs, are highly conserved, so that APP-binding proteins such as FE65, X11L, and JIPs interact with APLP1 and APLP2 as they do with APP (*14*, *17*, *24*). Therefore, we examined the binding of hARD1 to the cytoplasmic domains of APLP1 and APLP2. The pB42AD-hARD1 prey vector was co-transfected with pGilda bait vector including the cytoplasmic domain of APLP1 (APLP1cyt) and APLP2 (APLP2cyt) and monitored by growth on selective agar plates (Fig. 2B). hARD1 interacted with APLP1cyt and APLP2cyt as well as with APPcyt. The consensus sequence in the hARD1-binding site among the APP family proteins is -H-G-V/I-V-E-V-D-.

We then analyzed the region of hARD1 required for its interaction with APP. cDNAs encoding various hARD1 constructs in pB42AD prey vectors were co-transfected with pGilda-APPcyt bait vector (Fig. 2C). Only full-length hARD1 interacted with APPcyt. The deletion constructs lacking the carboxyl-terminal 50 (ΔC50), 100 (ΔC100), and 150 (ΔC150) amino acids lost the ability to bind APPcyt, which suggests that the C-terminal region of hARD1 contains the site that binds to APP. This result seems reasonable, because the amino-terminal ~160 amino acids contain the enzyme domain (Fig. 1A), although we cannot rule out the possibility that the carboxyl sequence may be critical for the maintenance of overall structure of hARD1.

We confirmed the interaction of APP with hARD1 in mammalian cells with a co-immunoprecipitation assay. FLAG-APP695 and HA-hARD1 were over-expressed in HEK293 cells, and the proteins were immunoprecipitated from cell lysates with anti-FLAG and anti-HA antibodies. The immunoprecipitates were analyzed by Western blotting with both antibodies. The anti-FLAG antibody recovered FLAG-APP together with HA-hARD1, and the anti-HA antibody recovered HA-hARD1 together with FLAG-APP (Fig. 3A). Immunostaining of cells expressing FLAG-APP695 and HA-hARD1 indicated that APP and hARD1 colocalized, especially in the peripheral region of the plasma membrane (Fig. 3B). These observations support the interaction of these proteins in the cell.

The hARD1-binding sequence was further examined in cells expressing HA-hARD1 and FLAG-tagged internal deletion constructs of APP (Fig. 3C). The FLAG antibody failed to co-immunoprecipitate HA-hARD1 when the APP constructs Δ657–664 and Δ653–664 were immunoprecipitated. Another internal deletion construct, Δ653–656, did co-immunoprecipitate with HA-hARD1. Amino acid substitutions of Ala for Tyr653 (Y653A) and Ilu656 (I656A), and their double substitution (Y653A/I656A) did not affect the interaction of FLAG-APP with HA-hARD1 in the cells (Fig. 3D). These results agree with the observations from our yeast two-hybrid assay (Fig. 2) and confirm that hARD1 associates with the novel motif 658-HGVVEVD-664 but not with known motifs such as 653-YTSI-656 and 681-GYENPTY-687.

N-Acetyltransferase Activity of the hADR1: hNAT1 Complex—Yeast ARD1 and NAT1 form a complex that expresses enzymatic activity (*30*). Because the endogenous substrate(s) has not been determined in yeast, the human ACTH peptide has been used as the conventional

substrate for amino-terminal acetylation by the ARD1:NAT1 complex (*28*). We therefore used the human ACTH peptide in an enzyme assay of the hARD1:hNAT1 complex. hARD1-HA and hNAT1-FLAG were expressed in HEK293 cells, the protein complex was recovered by immunoprecipitation with an anti-FLAG antibody, and the ACTH acetylation activity of the immunoprecipitate was examined (Fig. 4, upper panel). The immunoprecipitate from cells expressing both hARD1-HA and hNAT1-FLAG showed strong amino-terminal transferase activity (lane 4). The immunoprecipitate from cells expressing hNAT1-FLAG alone also showed moderate enzyme activity (lane 3), while that from cells expressing hARD1-HA alone did not show the activity (lane 2).

We also examined protein expression of proteins and complex formation by Western blotting of cell lysates and immunoprecipitates (Fig. 4, lower panel). The FLAG antibody immunoprecipitated hARD1-HA together with hNAT1-FLAG (lane 4), showing that hARD1 and hNAT1 form a complex in cells. Considering that HEK293 cells express endogenous hARD1 (data not shown), it is also possible that the FLAG antibody immunoprecipitates endogenous hARD1 together with hNAT1-FLAG in the absence of exogenous hARD1-HA expression, which would explain why the immunoprecipitate from cells expressing hNAT1-FLAG alone also had moderate enzyme activity.

A consensus sequence, including amino acid residues critical for the expression of enzyme activity, has been identified in the acetyl-CoA binding domains of yeast ARD1 and MAK3, another acetyltransferase (*35*, *36*). The consensus amino acid sequence is R-x-x-G-x-A, which is conserved in hARD1 (Fig. 5A). We prepared mutant constructs substituting Ala for Arg82 (hARD1^{R82A}) or for Gly85 (hARD1^{G85A}), and expressed these with hNAT1 in cells to examine the enzyme activity (Fig. 5B). Acetyltransferase activity was lower in lysates prepared from cells expressing hARD1^{R82A} or hARD1^{G85A} than from cells expressing wild-type hARD1. The hARD1^{R82A} mutant lost more enzyme activity than the hARD1^{G85A}. The result indicates that the consensus motif R-x-x-G-x-A is essential for hARD1 enzyme activity, as it is for yeast ARD1.

Effects of hARD1 and hNAT1 on Aβ Production—Many APP-binding proteins can modulate APP metabolism, including Aβ production (*21–26*). We examined whether hARD1 can also modulate Aβ secretion. Conditioned medium from N2a cells expressing APP with or

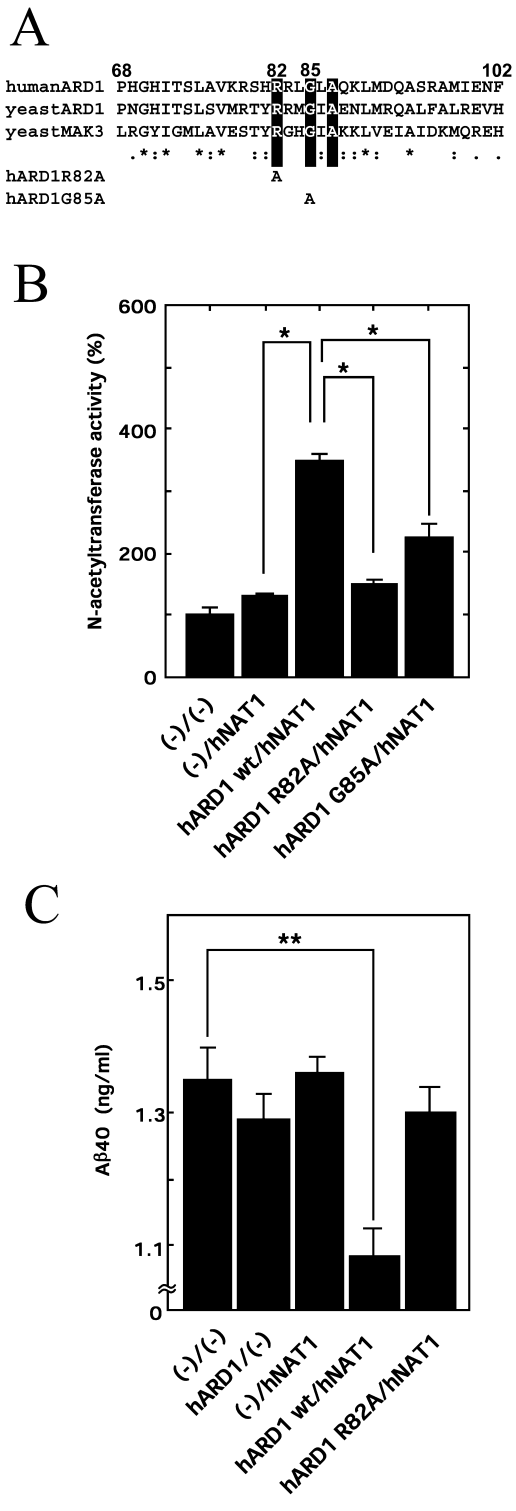

Fig. 5. **Relationship between N-acetyltransferase activity and Aβ40 secretion.** A: Amino acid sequence of region including of acetyl-CoA binding domain of hARD1, yeast ARD1 and yeast MAK3. The conserved amino acids in the consensus motif R-x-x-G-x-A are boxed. The asterisk (*) indicates the positions of identical amino acids, and the colon (:) and dot (.) indicate the positions of conservatively substituted amino acid residues, respectively. Amino acid substitutions for Arg82 (hARD1^{R82A}) and Gly85 (hARD1^{G85A}) of hARD1 used in this study are indicated. **B.** N-acetyltransferase activity of cells expressing hARD1 protein construct with hNAT1. HEK293 cells were transfected with pcDNA3-hARD1-HA constructs in the presence of pcDNA3-hNAT1-FLAG. To standardize the plasmid amounts, pcDNA3 vector (–) was added. The cell lysates were assayed for N-acetyltransferase activity, and the values are represented relative to the reference value of no transfection [(–)/(–)], set to 100%. The error bar indicates SEM (n = 3). *Asterisk* indicates statistical significance by ANOVA (*p < 0.05). C: Effect of hARD1 and hNAT1 expression on Aβ40 secretion from APP. N2a cells were transiently transfected with pcDNA3APP695 in the presence or absence of pcDNA3-hNAT1-FLAG, pcDNA3-hARD1 or both. The culture medium was collected and quantified for Aβ40 and Aβ42 (data not shown) using a sandwich ELISA. Results are the average of independent assays (n = 4). The error bar indicates SEM. Asterisk indicates statistical significance by ANOVA (**p < 0.005).

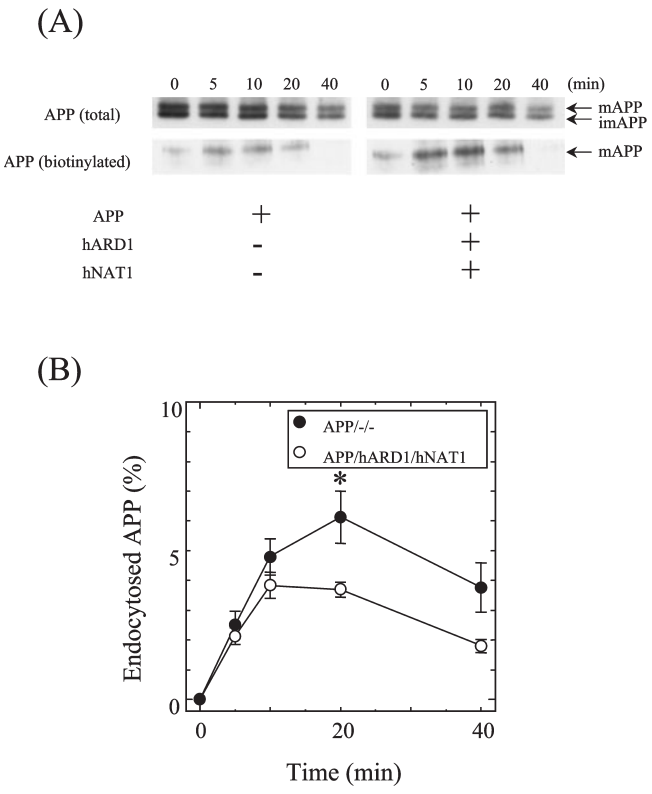

Fig. 6. **Suppression of APP endocytosis from cell surface by expression of hARD1 and hNAT1.** A: Cell-surface proteins of HEK293 cells (~1×10^7 cells) expressing FLAG-APP in the presence or absence of hARD1 and hNAT1 were biotinylated. After incubation for indicated times (0–40 min), cells were lysed after removal of cell-surface biotin, and the biotinylated protein was recovered with streptavidin agarose. The cell lysate (upper panel) and biotinylated protein were detected by Western blotting with anti-APP antibody UT18. B: Ratios (%) of biotinylated APP *versus* total cellular APP in lysate are indicated as the mean with S.D. (n = 3). mAPP, mature APP695; imAPP, immature APP695. *Asterisk* indicates statistical significance by standard t test (*$p < 0.05$).

without hNAT1, hARD1 or both was assayed for the amount of secreted Aβ. The expression of hARD1 or hNAT1 alone did not remarkably affect Aβ40 secretion (Fig. 5C). However, coexpression of both hARD1 and hNAT1 significantly suppressed Aβ40 secretion (~20%). When the partner of hNAT1 was altered to hARD1^{R82A} instead of wild-type hARD1, the ability to suppress Aβ40 secretion was lost. hARD1^{G85A} was less effective than hARD1^{R82A} in blocking the suppression of Aβ40 secretion. Expression of either or both of these proteins did not have a significant effect on Aβ42 secretion (data not shown). The observations suggest that the suppression of Aβ generation from APP needs N-acetyl transferase activity. An identical result was obtained when HEK293 cells were used (data not shown).

Possible Mechanism of Aβ Suppression by hARD1 and hNAT1—To understand the mechanism of Aβ suppression in cells expressing hARD1 and hNAT1, we performed APP endocytosis assay, because Aβ40 is generated in the early secretory and endocytic pathways (*37*) and the cytoplasmic domain of APP plays an important role in APP endocytosis (*20*). Cell-surface proteins were biotinylated, and APP endocytosis was assayed in HEK293 cells expressing APP in the presence or absence of hARD1 and hNAT1 (Fig. 6). During 20 min of incubation following biotinylation, approximately 6% of total cellular APP was detected in biotinylated forms which indicates internalized APP, while only 3–4% of APP was subjected to endocytosis in the presence of hARD1 and hNAT1. This suppression of endocytosis of APP by expression of hARD1 and hNAT1 is likely to correlate with the decreased Aβ40 generation by expression of hARD1 and hNAT1.

DISCUSSION

It is widely believed that the production, secretion, and aggregation of Aβ are the first steps in the neural cell death that results in the onset of AD (*1–3*). APP, the precursor of Aβ, is a type I membrane protein whose metabolism and functions are regulated by its cytoplasmic domain (*4–26*). APPcyt is reported to contain at least three functional motifs, 653-YTSI-656, 667-VTPEER-672, and 681-GYENPTY-687 (numbering for APP695 isoform). In particular, many cytoplasmic proteins such as FE65, X11s, and JIPs interact with the 681-GYENPTY-687 motif (*21–26*). We found that hARD1 associates with a novel motif in APP, 658-HGVVEVD-664. This is the first report of an *N*-acetyltransferase associating with APP. Moreover, hARD1 gained enzymatic activity when it formed a complex with hNAT1. Interestingly, the enzyme activity correlated with the suppression of Aβ40 secretion derived from APP, and amino acid substitutions of hARD1 resulting in the loss of enzyme activity also caused the Aβ40 suppression to be lost, despite normal binding to APP and hNAT1. We further found that endocytosis of cell-surface APP is suppressed in the presence of hARD1 and hNAT1. This effect may cause the decreased secretion of Aβ40, because Aβ40 is believed to be generated in the early secretory and endocytic pathways (*37*), although the detailed mechanism of the suppression of Aβ40 secretion by N-acetyltransferase is still under investigation.

Recent studies have elucidated the functions of mammalian N-acetyltransferase. Jeong *et al.* found that ARD1-mediated acetylation of hypoxia-inducible factor 1 (HIF-1) enhances stabilization of HIF-1 metabolism (*38*), and Sugiura *et al.* reported that mouse NAT1 may play an important role in neural generation and differentiation (*39*). These observations, together with our findings, suggest that the mammalian N-acetyltransferase composed of hARD1 and hNAT1 may contribute to the stabilization of targeted proteins. The substrate protein of N-acetyltransferase in our system is still unknown. One possibility may be other APP-binding proteins. hARD1 interacts with the 658-HGVVEVD-664 motif, and its binding therefore does not compete with the APP-binding of other cytoplasmic proteins, such as FE65, X11s, and JIP, which associate with the 681-GYENPTY-687 motif. However, we have not demonstrated whether these known APP-binding proteins are subject to N-terminal acetylation. The APPcyt may work as a scaffold between the hARD1:hNAT1 complex and APP-binding proteins. Another possibility is that AICD itself may be a substrate of the hARD1:hNAT1 complex. A recent report indicated

that AICD plays an important role in gene transactivation, but it is known that AICD is very unstable in cells if it cannot associate with FE65 (*40*). Thus, the amino-terminal acetylation of AICD by binding of the hARD1: hNAT1 complex may contribute to the intracellular stabilization of AICD. Although further studies are needed to analyze the significance of the association of APP and hARD1 and to identify the intracellular substrate of the hARD1:hNAT1 complex, our findings may shed light on a new approach for analyses of APP metabolism and function in AD pathogenesis.

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