Autosomal Recessive Hypercholesterolemia Protein Interacts with and Regulates the Cell Surface Level of Alzheimer’s Amyloid β Precursor Protein*

Cristiana Noviello‡‡, Pasquale Vito‡‡, Peter Lopez, Mona Abdallah***, and Luciano D’Adamio‡‡‡

From the ‡‡ Albert Einstein College of Medicine, Department of Microbiology & Immunology, Bronx, New York 10461, §CEINGE-Biotechneologica Avanzate, Università degli Studi Federico II, Naples 80131, †Dipartimento di Scienze Biologiche ed Ambientali, Università degli Studi del Sannio, Benevento 82100, Italy, ‡‡‡ Aaron Diamond AIDS Research Center, Rockefeller University, New York, New York 10016, and ‡‡‡‡Zymed Laboratories, South San Francisco, California 94080

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The familial Alzheimer’s disease gene product amyloid β protein precursor (AβPP) is sequentially processed by β- and γ-secretases to generate the Aβ peptide. Although much is known about the biochemical pathway leading to Aβ formation, because extracellular Aβ aggregates of Aβ peptides are considered the cause of Alzheimer’s disease, the biological role of AβPP processing is only recently being investigated. Cleavage of AβPP by γ-secretase releases, together with Aβ, a COOH-terminal AβPP intracellular domain, termed AID. Hoping to gain clues about proteins that regulates AβPP processing and function, we used the yeast two-hybrid system to identify proteins that interact with the AID region of AβPP. One of the interactors isolated is the autosomal recessive hypercholesterolemia (ARH) adapter protein. This molecular interaction is confirmed in vitro and in vivo by fluorescence resonance energy transfer and in cell lysates. Moreover, we show that reduction of ARH expression by RNA interference results in increased levels of cell membrane AβPP. These data assert a physiological role for ARH in AβPP internalization, transport, and/or processing.

AβPP1 is a ubiquitous type I transmembrane protein that undergoes extensive proteolytic processing along two major pathways (1–5). In the amyloidogenic pathway, AβPP is cleaved in the ectodomain by the β-secretase forming a C99 membrane-bound intermediate. C99 can be cleaved by the γ-secretase to release Aβ and the APP intracellular domain (AID). Alternatively, AβPP can be cleaved within the Aβ sequence by the α-secretase creating a C33 membrane-bound intermediate which produces P3 and AID after γ-cleavage. AβPP processing is firmly associated with the pathogenesis of Alzheimer’s disease (AD) because mutations associated with familial forms of AD are found in AβPP itself and in the highly homologous genes PS1 and PS2, which are key components of a multimolecular complex with γ-secretase activity (6–15).

Although the role of Aβ peptides in the pathogenesis of AD has been extensively studied, reports as to the role of AID are very recent. AID-like peptides have been identified in human brains from cases of sporadic AD (16) and play a role in apoptosis (16), transcription (17–20), and Ca2+-release from the endoplasmic reticulum (21).

AβPP processing and AID signaling can be regulated by AβPP-interacting proteins (17–22). Thus, to find AβPP-binding proteins we employed the yeast two-hybrid selection system. This screening resulted in the identification of several proteins that bind the intracellular domain of AβPP. Here we report the novel AβPP interactor ARH, an adapter protein that has been shown to regulate cholesterol uptake by genetic studies (22). These data suggest that ARH may be a mediator of the well described effect of cholesterol metabolism on AβPP processing.

MATERIALS AND METHODS

Yeast Two-Hybrid System—The two-hybrid screening was conducted using the Matchmaker system from Clontech according to the manufacturer’s instruction. For library screening, Yeast190 expressing GAL4BD-AID fusion proteins were transformed with a human fetal brain cDNA library cloned in the pACT2 vector (Clontech). 2 × 106 colonies were analyzed. Transformed yeast were selected in synthetic drop-out plates lacking tryptophan, leucine, and histidine in the presence of 50 μg/ml 3-aminoazonole (Sigma) and grown for 5 days at 30 °C. Colonies growing on selective media were scored as positive. Assays were done for eight independent transformants.

cDNA Cloning and Constructs—The GAL4BD-AβPP bait was constructed using the psA2 vector (Clontech) and consisted of the COOH-terminal amino acids of AβPP fused to the DNA binding domain of GAL4, respectively, AβPP, AβPPNcas, and AID were made as described previously (16). GST fusion proteins were made in pGEX vectors (Amersham Biosciences). Mutations were introduced by using the transformer site-directed mutagenesis kit (Clontech).

ARH, ARHt (clone AT60), ARHPTB (amino acid 48–175), and ARHPIPTB (amino acids 170–308) were cloned into pECFP-N1 (Clontech), pcDNA3.1, and FLAG-tagged pcDNA3.1 (Invitrogen) for expression in mammalian cells or in vitro. Fe65, Fe65N-PTB, and Fe65C-PTB were cloned in FLAG-pcDNA3. All constructs were confirmed by sequencing.

Cell Lines and Transfections—Human embryonic kidney (HEK) 293T cells were grown in RPMI 1640 media (Invitrogen) supplemented with 10% horse inactivated fetal calf serum (Biofluids; Rockville, MD). Transfections were performed in 6-well plates either using Metafectene (Bionext Laboratories GmbH) with 3 μl per 1 μg of DNA.

Northern Blot Analysis—A multistain blot was purchased from Clontech, and it was hybridized with a 32P-labeled ARHPTB probe following the manufacturer’s instruction. After washing, the blot was developed by autoradiography.

GST Pull-down, Immunoprecipitation, and Immunoblot Analysis—
performed overnight at 4 °C for 10 min. Some lysate representing the total lysate was harvested between 18 and 24 h after transfection in their conditioned media (phosphate-buffered saline, 0.5 mM EDTA, washed once with FACS media (phosphate-buffered saline, 2% fetal bovine serum), and stained for 1 h at 4 °C in rotation with 2.5 μg/ml P2-1 antibody (BIOSOURCE 44-100) or with the IgG1 isotype control antibody P3 (gift of Dr. Porcelli). Cells were rinsed twice with FACS media and stained with Red-phyceroerythrin-conjugated secondary antibody (Southern Biotech 1030-09). Cells were analyzed by FACSCalibur (BD Biosciences).

RESULTS AND DISCUSSION

To identify proteins interacting with the cytoplasmic domain of AβPP, we have used the yeast two-hybrid selection system. This screening resulted in the identification of two independent clones coding for a novel protein (the interaction in yeast between AID and the AT60 clone is shown in Fig. 1a). Northern blot analysis showed that AT60 is expressed, albeit at different levels, in all human tissues analyzed (Fig. 1b). A recent blast search showed that AT60 is identical to the recently identified ARH adapter protein (22) (Fig. 1c).

ARH Interacts with AβPP

Recombinant GST fusion proteins were expressed in Escherichia coli strain BL21 (Invitrogen) to make non-phosphorylated proteins and strain TKH1 (Strategene) to make tyrosine-phosphorylated proteins using the pfGx system (Amersham Biosciences) (23). Proteins were purified using glutathione-Sepharose beads.

For GST pull down of proteins produced in vitro (Fig. 2a), [3H]leucine-labeled proteins were made using the Tnt-coupled in vitro transcription/translation system (Promega). After synthesis of the radiolabeled protein for 1.5 h, aliquots of the proteins were incubated with GST fusion proteins bound to glutathione-Sepharose beads for 2 h at room temperature. The beads were then washed three times with lysis buffer T (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 45 mM NaCl) and boiled with SDS loading buffer with DTT. The proteins were separated by SDS-PAGE, and the gel were fixed with 50% methanol, 40% H2O, 10% acetic acid. The gel was incubated in Amplify (Amersham Biosciences) for 20 min and dried, and signals were detected using autoradiography.

For GST pull down of proteins produced in vitro (Fig. 2b), HEK293 cells were lysed in lysis buffer T containing a protease inhibitor tablet (Roche Applied Science) 24–48 h following transfection. Lysis was allowed to continue for 10 min on ice and was then spun down at full speed at 4 °C for 10 min. Some lysate representing the total lysate was removed and boiled with SDS loading buffer with DTT, whereas the rest was pulled down with GST fusion proteins. The beads were washed five times with lysis buffer and boiled with SDS loading buffer with DTT. The proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Bio-Rad). Membranes were probed with either αFLAG or rabbit polyclonal αARH (Zymed Laboratories Inc., new product catalog number 36-0400) followed by horseradish peroxidase-conjugated secondary antibodies (Southern Biotech). Proteins were detected using the Supersignal West Pico chemiluminescent system (Pierce).

For immunoprecipitation from transfected cells, lysates were immunoprecipitated for 2 h at room temperature with the αFLAG monoclonal antibody bound to agarose beads (Sigma). After washing and SDS-PAGE, membranes were probed with either the 22C11 monoclonal antibody directed against the ectodomain of AβPP (Calbiochem) or rabbit polyclonal αARH.

For co-immunoprecipitation of endogenous proteins, untransfected HEK293 cells were lysed as above. One mg of protein was used for immunoprecipitation with rabbit polyclonal αAPP COOH-terminal (Zymed Laboratories Inc., new product), rabbit polyclonal αARH, or rabbit anti-mouse IgG antibodies (ICN, Aurora, OH). Immunoprecipitations were performed overnight at 4 °C, followed by incubation of immunoprecipitates with protein A-agarose beads, washing, and immunoblot, as described above.

FRET Analysis—HEK293T cells were plated in 24-well plates and co-transfected with the CFP and YFP fusion proteins using Metafectene. All transfections contained ratios between the two cDNAs determined empirically to yield the best co-expression as follows. Cells were

RNA Interference—HEK293T cells were plated in 24-well plates and transfected with the following small interfering (si) RNA duplex using TransIT-TKO (Mirus): Scramble (CAGUCCGCUUUGACUGGdTdT and CCAUGCGAACCGACUAGdTdT); ARH (GCCUUGAGUGCGACGUAGdTdT and CACUGUGCCCACUAAGGGdTT); and AβPP (CUUGACUGACUCGCGGdTTT and CAUGCGUCGAUCGGAGdTdT). FACS Analysis—For FACS analysis HEK293 cells were collected with phosphate-buffered saline, 0.5 mM EDTA, washed once with FACS media (phosphate-buffered saline, 2% fetal bovine serum), and stained for 1 h at 4 °C in rotation with 2.5 μg/ml P2-1 antibody (BIOSOURCE 44-100) or with the IgG1 isotype control antibody P3 (gift of Dr. Porcelli). Cells were rinsed twice with FACS media and stained with Red-phyceroerythrin-conjugated secondary antibody (Southern Biotech 1030-09). Cells were analyzed by FACSCalibur (BD Biosciences).
plexes were purified, resolved by gel electrophoresis, and visualized by autoradiography. Fig. 2a shows that GST-AID specifically and directly binds both ARHPTB and Fe65C-PTB but not Fe65N-PTB. Next, HEK293 cells were transfected with constructs expressing either ARH or ARHΔPTB (a mutant lacking the PTB domain) of FLAG-tagged Fe65. Cell lysates were incubated with GST-AID, GST-AID phosphorlyated on Tyr$^{682}$ (GST-AID(t)), GST-AID$^{Y682C}$ (in which tyrosine$^{682}$ was mutated to glycine), or GST, and pull downs were resolved by gel electrophoresis. Western blot analysis (Fig. 2b) using either aARH or aFLAG antibodies revealed that ARH, like Fe65, interacted with AID, whereas ARHΔPTB did not. We also found that, similarly to Fe65, AβPP binds ARH independent of Tyr$^{682}$ phosphorylation, but this binding is abolished by the Y682G mutation.

In further experiments, HEK293 cells were co-transfected with FLAG-ARH and the following AβPP constructs: wild type AβPP, AβPPNcas (an AβPP mutant lacking the COOH-terminal 31 amino acids that include the YENPTY motif), AβPPY682F (in which Tyr$^{682}$ was mutated to phenylalanine), AβPPY687A (in which Tyr$^{687}$ was mutated to alanine), or AβPP$^{Y682F}$ (in which Thr$^{683}$ was mutated to phenylalanine). Cell lysates were immunoprecipitated with aFLAG monoclonal antibody and analyzed by Western blot. Fig. 3a shows that although FLAG-ARH immunoprecipitates AβPP, it does not immunoprecipitate AβPPNcas which lacks the YENPTY motif. Additionally, AβPP$^{Y682F}$ mutation significantly affects the interaction with ARH.

To detect interaction between ARH and AβPP in living cells, we used fluorescence resonance energy transfer (FRET) (25–27). HEK293T cells were co-transfected with yellow fluorescent protein-AID (Y-AID) and either cyan fluorescent protein-ARH (C-ARH) or C-ARHΔPTB fusion proteins. In FRET on living cells, if the proteins are in close proximity, on the order of 10 nm or less, the energy from the excitation of CFP will be transferred to YFP, and emission at the wavelength of YFP will be detected. If the proteins are not within this proximity, excitation of CFP is not transferred, and only emission at the wavelength of CFP will be detected. Importantly, FRET was detected when Y-AID was co-transfected with C-ARH but not with C-ARHΔPTB. Altogether, these data indicate that ARH interacts with the YENPTY motif of AβPP through its PTB domain. Furthermore, this interaction is independent of Tyr$^{682}$ phosphorylation but requires the presence of Tyr$^{682}$.

To determine whether endogenous AβPP and ARH interact, we immunoprecipitated HEK293 lysates either with an aAβPP or an aARH polyclonal antibody. As shown in Fig. 4, which is representative of data from two independent experiments, AβPP was immunoprecipitated with both aAβPP and aARH antibodies, whereas AβPP was not immunoprecipitated with a control rabbit anti-mouse IgG antibody (RαM). Similar findings were obtained with the reverse experiment, that is ARH was
in vivo

immunoprecipitated by the αβPP and the αARH antibodies but not by the RoM IgG control. Altogether, these experiments indicate that endogenous αβPP and ARH associate.

To determine whether ARH physiologically regulates αβPP biology, we have repressed ARH protein expression in HEK293 cell using RNA interference (RNAi). Conversely, reduction of ARH protein levels results in increase of cell surface αβPP. This experiment is representative of three independent experiments. Sc., scrambled sequence.

Fig. 4. ARH depletion results in increased cell membrane αβPP levels. a, HEK293 cells were transfected with the indicated siRNAs and analyzed by Western blot with the indicated antibodies 48 h later. b, endogenous αβPP expressed on the cell surface of HEK293 cells can be detected and quantitated using the anti-αβPP monoclonal antibody P2-1 in FACS analysis. The isotype control P3 does not stain these cells, and overexpression of AβPP in FACS analysis. The isotype control P3 does not stain these cells, and overexpression of AβPP (Zymed Laboratories Inc. anti-COOH-terminal AβPP domain), and αARH antibodies. Western blotting (W.B.) was done with either αARH or αβPP ectodomain antibody 22C11.

Fig. 5. ARH depletion results in increased cell membrane αβPP levels. a, HEK293 cells were transfected with the indicated siRNAs and analyzed by Western blot with the indicated antibodies 48 h later. b, endogenous αβPP expressed on the cell surface of HEK293 cells can be detected and quantitated using the anti-αβPP monoclonal antibody P2-1 in FACS analysis. The isotype control P3 does not stain these cells, and overexpression of AβPP (HEK293-αβPP) increases P2-1 staining. c, the levels of cell membrane αβPP are decreased in cells depleted of AβPP by RNAi. Conversely, reduction of ARH protein levels results in increase of cell surface αβPP. This experiment is representative of three independent experiments. Sc., scrambled sequence.

antibody, cells were incubated with a phycoerythrin-labeled anti-mouse IgG secondary antibody and analyzed by FACS. P2-1 specifically binds to the cell surface AβPP of HEK293 cells as determined by three complementary facts: (i) the isotype-matched P3 antibody does not bind HEK293 cells (Fig. 5b); (ii) overexpression of AβPP in HEK293 cells increases P2-1 binding (Fig 5b); (iii) conversely, reduction of AβPP protein levels by RNAi results in decreased P2-1 binding (Fig 5c). Of interest, reduction of ARH protein levels by RNAi results in increased amounts of AβPP on the cell membrane of HEK293 cells (Fig 5c). These data indicate that ARH physiologically regulates the cell surface level of AβPP.

In this study we have demonstrated in vitro, in vivo, in living cells, and for endogenous proteins the interaction between AβPP and ARH. Moreover, we have shown that ARH regulates AβPP cell membrane levels. These changes in AβPP cell surface levels in ARH-low cells reflect a physiological role for ARH in either AβPP internalization, transport to the cell membrane, and/or shedding of the ectodomain by secretases (α and/or β). Although further work will be required to discriminate among these possibilities, our data nevertheless prove a function for ARH in AβPP biology.

Genetic defects in ARH impair endocytosis of low density lipoprotein receptors family members (22, 33, 34), thereby reducing cholesterol uptake and increasing plasma concentration of cholesterol. Of interest, biochemical, epidemiological, and genetic evidence have involved cholesterol metabolism in the regulation of AβPP processing and the pathogenesis of AD (35–38). The finding that ARH interacts with AβPP and regulates the cell membrane levels of AβPP is provocative and entices us to speculate that ARH may be a molecular link between AβPP processing and cholesterol.

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