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Alzheimer's Disease-related Overexpression of the Cation-dependent Mannose 6-Phosphate Receptor Increases Aβ Secretion

ROLE FOR ALTERED LYSOSOMAL HYDROLASE DISTRIBUTION IN β-AMYLOIDOGENESIS*

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Paul M. Mathews‡§¶, Carolyn B. Guerra‡‖, Ying Jiang‡, Olivera M. Grbovic‡, Benjamin H. Kao, Stephen D. Schmidt‡, Ravi Dinakar‡, Marc Mercken‡‡, Annette Hille-Rehfeld§§, Jack Rohrer¶¶, Pankaj Mehta‖‖, Anne M. Cataldo**, and Ralph A. Nixon‡§**

*From the ‡Nathan Kline Institute and §New York University School of Medicine, Orangeburg, New York 10962, **McLean Hospital, Harvard Medical School, Belmont, Massachusetts 02478, ‡‡Janssen Research Foundation, B-2340 Beerse, Belgium, §§Center for Biochemistry and Molecular Cell Biology, University of Göttingen, D-37073 Göttingen, Germany, ¶¶Friedrich Miescher Institut, 4058 Basel, Switzerland, and ‖‖New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York 10314*

Prominent endosomal and lysosomal changes are an invariant feature of neurons in sporadic Alzheimer's disease (AD). These changes include increased levels of lysosomal hydrolases in early endosomes and increased expression of the cation-dependent mannose 6-phosphate receptor (CD-MPR), which is partially localized to early endosomes. To determine whether AD-associated redistribution of lysosomal hydrolases resulting from changes in CD-MPR expression affects amyloid precursor protein (APP) processing, we stably transfected APP-overexpressing murine L cells with human CD-MPR. As controls for these cells, we also expressed CD-MPR trafficking mutants that either localize to the plasma membrane (CD-MPRpm) or to early endosomes (CD-MPRendo). Expression of CD-MPR resulted in a partial redistribution of a representative lysosomal hydrolase, cathepsin D, to early endosomal compartments. Turnover of APP and secretion of sAPPα and sAPPβ were not altered by overexpression of any of the CD-MPR constructs. However, secretion of both human Aβ40 and Aβ42 into the growth media nearly tripled in CD-MPR- and CD-MPRendo-expressing cells when compared with parental or CD-MPRpm-expressing cells. Comparable increases were confirmed for endogenous mouse Aβ40 in L cells expressing these CD-MPR constructs but not overexpressing human APP. These data suggest that redistribution of lysosomal hydrolases to early endocytic compartments mediated by increased expression of the CD-MPR may represent a potentially pathogenic mechanism for accelerating Aβ generation in sporadic AD, where the mechanism of amyloidogenesis is unknown.

Deposition in the brain parenchyma and cerebral vessel walls of β-amyloid and intracellular neurofibrillary tangles are diagnostic hallmarks of AD.[1] β-Amyloid consists primarily of the short peptide Aβ, which is derived from the proteolytic cleavage of the amyloid precursor protein (APP) at a site within its luminal domain (the β-cleavage site) and cleavage within the transmembrane domain (the γ-cleavage site). Additionally, α-cleavage of APP may occur within the Aβ domain of APP, adjacent to the plasma membrane (see Ref. 1 for a review of APP proteolytic processing). β-Amyloidogenesis in familial AD caused by mutation of APP or the presenilins involves, at least in part, the overproduction of Aβ (2); however, the mechanisms promoting β-amyloidogenesis in sporadic forms of AD, which account for >90% of AD cases, remain unclear.

Experimental evidence has shown that early endosomes are an important site for APP processing, including the generation of Aβ. Expression of trafficking mutants of APP lacking endocytosis signals reduces Aβ production compared with endocytosis-competent wild-type APP (3, 4). There is also evidence that at least some of the proteases responsible for Aβ generation reside within early endosomes. For example BACE, a recently identified transmembrane aspartic protease with β-secretase activity, resides in part within early endosomes (5–7). The lysosomal protease cathepsin D (Cat D) was recently confirmed to have secretase-like activity (8) and, as discussed below, can be detected in early endosomes, particularly in the abnormally enlarged early endosomes of neurons from AD brain (9). Finally, we have detected Aβ immunoreactivity within the abnormally enlarged early endosomes seen in neurons of individuals with Down syndrome and AD.[2]

Neuronal endocytic abnormalities precede substantial β-amyloid deposition in AD brain and appear decades prior to the development of AD pathology in Down syndrome (10). Abnormalities of the endocytic pathway in AD are characterized by increased early endosome volume, increased expression of proteins involved in the regulation of endocytosis, endosomal fusion, and recycling (such as Rab5, rabaptin, and Rab4), and abnormally increased levels of lysosomal hydrolases within the early endosome (9). Lysosomal system activation, which develops in virtually all neurons within cell populations that are potentially vulnerable to the disease process, is characterized

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¶ To whom correspondence should be addressed. Tel.: 845-398-5421; Fax: 845-398-5422; E-mail: mathews@nki.rfmh.org.

‖ Current address: Dept. of Research, Alfred I. duPont Hospital for Children, Wilmington, DE 19803.

[1] The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein; sAPP, soluble APP; Cat D, cathepsin D; MPR, mannose 6-phosphate receptor; CD-MPR, cation-dependent mannose 6-phosphate receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; EEA1, early endosome antigen 1; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; BACE, beta-site APP-cleaving enzyme.

[2] A. M. Cataldo, unpublished observation.

by increased gene expression and protein expression for all classes of lysosomal hydrolases, including proteases such as Cat D, and a robust proliferation of lysosomes (11–14). Additionally, Cat D levels in the cerebrospinal fluid are increased in individuals with sporadic AD, suggesting that changes in the intracellular trafficking of lysosomal enzymes result not only in their abnormal accumulation within early endosomes but also in their secretion from neurons (15).

A possible mechanism underlying the partial redistribution of lysosomal hydrolases in AD brain is a change in the expression of neuronal mannose 6-phosphate receptors (MPR). The two MPRs bind the majority of lysosomal hydrolases in the trans-Golgi network via the mannose 6-phosphate recognition signal and mediate their transport to organelles within the endosomal-lysosomal system (16). Cataldo *et al.* (9) demonstrated by immunocytochemistry an increased neuronal expression of the 46-kDa, cation-dependent MPR (CD-MPR) in sporadic AD cases when compared with control. This increase in CD-MPR immunoreactivity in sporadic AD brain was not accompanied by increased levels of the cation-independent MPR (CI-MPR), a ~300,000-kDa transmembrane glycoprotein containing two mannose 6-phosphate binding sites as well as a binding site for insulin-like growth factor II (16, 17). Although both the CD-MPR and CI-MPR can divert the majority of mannose 6-phosphate-tagged hydrolases from the secretory pathway to the endosomal-lysosomal system, it is now clear that these receptors are not functionally redundant (18, 19). The CD-MPR preferentially delivers newly synthesized lysosomal hydrolases to endocytic compartments, including the early endosome (20), which may in part explain the increased localization of lysosomal hydrolases in neuronal early endosomes in AD (9).

In this study, we have modeled the increase in lysosomal hydrolases seen in early endosomes in sporadic AD by overexpressing human CD-MPR in a mouse fibroblast-like cell line. Overexpression of CD-MPR, which was localized in part to early endosomes, resulted in partial redistribution of a marker lysosomal protease, Cat D, to this compartment. Although this had little effect on rates of APP turnover and sAPP production, it had a dramatic impact on Aβ secretion, increasing Aβ levels in the growth media more than 2.5-fold. These results suggest that abnormalities of the protease composition within early endosomes in AD may contribute directly to AD pathogenesis by promoting Aβ generation and release early in the disease process and may have relevance to sporadic forms of AD in which the amyloidogenic mechanism has been elusive.

MATERIALS AND METHODS

Cell Lines and cDNA Constructs—Ltk cells (a murine fibroblast-like cell line (21)) were maintained at 37 °C and 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% fetal bovine serum (Gemini), 2 mM Glutamax I (Invitrogen) and penicillin/streptomycin (Cellgro). Following transfection using Lipofectin (manufacturer's protocol; Invitrogen), selection was made in 400 μg/ml G418 (Gemini), alone or combined with 200 μg/ml hygromycin B (Invitrogen) or hypoxanthine/aminopterin/thymidine (HAT medium; Invitrogen).

A cDNA encoding a 2.4-kb full-length CD-MPR was isolated from a human liver cDNA library (Stratagene) by standard techniques using PCR-generated cDNA fragments as probes. The construct CD-MPRendo was generated using PCR and an antisense oligonucleotide to replace the cytoplasmic tail CD-MPR following amino acid residue Tyr^{210} with the amino acid sequence NGSLQYRICI. Similarly, CD-MPRpm was generated using the oligonucleotide to introduce the amino acid sequence NGSLQCRICI at the same point in the CD-MPR molecule. These constructs, as well as a cDNA encoding human APP_{695}, were inserted into the mammalian expression vector pcDNA3 (Invitrogen). CD-MPR constructs were transfected into the same progenitor clone of APP_{695}-overexpressing L cells using co-transfection with a vector carrying hygromycin B resistance (CD-MPRendo using pCEP4) or the *tk* gene (CD-MPR and CD-MPRpm (22)).

Antibodies—Anti-cathepsin D affinity-purified antiserum was purchased from Santa Cruz Biotechnology. The monoclonal antibodies that specifically recognize the luminal/extracellular domain of human CD-MPR have been described previously (10C6 (23) and 22D4 (24–26)). Purified early endosome antigen 1 (EEA1) antiserum was kindly supplied by Dr. Silvia Corvera at the University of Massachusetts Medical School (27). The anti-APP luminal domain monoclonal antibody P2-1 was the generous gift of Dr. Maria Kounnas at Sibia (28), and monoclonal antibody 6E10 was purchased from Senetek. TGN38, a marker of the trans-most saccule of the Golgi apparatus, was detected with a sheep polyclonal antibody purchased from Serotec (Raleigh, NC) (29). Fluorescent secondary antibodies were purchased from Cappel (Durham, NC). The monoclonal antibody C1/6.1 was generated in our laboratory against a peptide corresponding to the carboxyl-terminal 20 amino acids of APP using standard protocols (30).

Preparation of Human Brain Homogenates and Western Blot Analysis—Human postmortem frozen brain tissue was obtained from the Harvard Brain Tissue Resource Center at McLean Hospital. Brain tissue was evaluated according to CERAD criteria (31) and the criteria proposed by Mirra *et al.* (32) and staged according to Braak and Braak (33). Control brains showed no evidence of neurofibrillary or plaque pathology. Postmortem tissue samples from 10 cases were diagnosed as having neuropathological evidence of early stage AD (neocortex devoid of neuritic plaques and transentorhinal, entorhinal cortex/hippocampus with sparse plaques; these are Braak stages I–III). A second group of five brains met the diagnosis of late stage AD using the above criteria and the NIA-Reagan guidelines for the diagnosis of AD (34) (>30 neuritic plaques/high power field in the entorhinal-perirhinal cortex, hippocampus, and other limbic regions, and association and visual cortices; Braak stages V–VI). Proteins were extracted from prefrontal cortex gray matter, region A10, in 10 volumes of 150 mM NaCl, 25 mM Tris pH 7.4, 1 mM EDTA, 0.5 mM iodoacetamide, 1% Triton X-100. Equal amounts of proteins were denatured in SDS sample buffer lacking any reducing agent and sized by SDS-PAGE prior to transfer to polyvinylidene difluoride membranes. CD-MPR was detected by overnight incubation in 22D4, and antibody binding was detected as described below.

Fluorescence Labeling of Cultured Cells—Cells were seeded onto glass coverslips, and expression of APP and the CD-MPR constructs was induced by the addition of 20 mM butyrate for ~48 h (22). Cells were gently fixed in 1% paraformaldehyde, 5% sucrose in phosphate-buffered saline (pH 7.4) at room temperature for 20 min, and immunolabeled as described previously (22). Cells were permeabilized by the addition of 0.1% saponin (Sigma) to the antibody-containing solutions. Alternatively, for cathepsin D, cells grown on coverslips were fixed by the addition of cold methanol for 15 min, washed, and immunolabeled as above.

For BODIPY-pepstatin A labeling, living cells were incubated at 37 °C in medium containing 5 mM BODIPY-pepstatin A (Molecular Probes), washed with cold Hank's balanced salt solution, and fixed in paraformaldehyde. Cells were mounted in Vectashield (Vector Laboratories) and examined by epifluorescence or confocal microscopy.

Subcellular Fractionation on Optiprep Gradients—Optiprep was purchased from Accurate Chemical Co. (Westbury, NY). Our protocol is modified from that recommended by the manufacturer and as described previously (35). Cells were seeded on to 100-mm-diameter plates, allowed to settle, and treated with butyrate for 48 h. To prepare the homogenate, cells were scraped in phosphate-buffered saline with protease inhibitors, pelleted, and resuspended in 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4) and homogenized with 10 strokes of a steel cell cracker. A post-nuclear supernatant was prepared by spinning for 15 min in an Eppendorf centrifuge and layered onto a gradient consisting of 1 ml each of 30, 25, 20, 15, 12.5, 9, 7.5, 5, 2.5% Optiprep diluted in 250 mM sucrose, 6 mM EDTA, 60 mM HEPES (pH 7.4). After centrifugation at 100,000 × *g* for 1 h, fractions of 1.1 ml were collected from the bottom, and equal protein was loaded onto SDS polyacrylamide gels.

Metabolic Labeling and Immunoprecipitations—To detect APP and sAPP, 5×10^5 cells were seeded onto 35-mm-diameter tissue culture dishes followed by induction with 20 mM butyrate for 24 h. Cultures were methionine/cysteine-starved for 20 min, pulse-labeled for 15 min with 100 μCi/ml Tran^{35}S-label (PerkinElmer Life Sciences), washed, and chased in complete medium containing 2 mM unlabeled methionine (22). Both the chase medium, briefly spun to remove cellular debris, and cell lysates (prepared in 1% Triton X-100, 140 mM NaC1, 25 mM Tris pH 7.4, 10 mM methionine, and protease inhibitors (22, 36)) were subjected

to immunoprecipitation as described under "Results." Immunoprecipitated proteins were sized by SDS-PAGE, and labeled proteins were visualized by exposure to x-ray film and analyzed quantitatively using a Storm 840 PhosphorImager (Molecular Dynamics).

Determination of Aβ40 and Aβ42 Levels by Sandwich ELISA and Western Blot Analysis of APP Levels—1.5×10^6 cells were seeded onto 100-mM-diameter tissue culture dishes and incubated for 40 h in medium containing 20 mM butyrate. Medium was replaced by fresh butyrate containing medium (2.5 ml/100-mM-diameter dish), and conditioned media were collected after 8 h. Conditioned media pooled from duplicate plates were spun briefly and frozen in aliquots in a dry ice/methanol bath prior to storage at −70 °C. Aβ40 and Aβ42 levels were measured by sandwich ELISA using 6E10 as the capture antibody and Aβ40 or Aβ42 carboxyl-terminal-specific detection antibodies as described previously (37, 38). Following collection of conditioned media, Triton X-100 lysates were prepared from each dish (22). The amount of total protein in cell lysates was determined (Bio-Rad DC protein assay) to confirm equal cell density, and APP was examined by Western blot analysis to confirm equal levels of APP expression (see Fig. 4). For Western blot analysis, lysates were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were incubated in primary antibody overnight, washed, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 1.5 h. Membranes were incubated in ECL substrate (Amersham Biosciences) and exposed to x-ray film.

Murine Aβ was detected by sandwich ELISA from similarly prepared media samples using Aβ40 and Aβ42 antibodies. Synthetic peptides were used to immunize mice for the production of monoclonal antibodies. Antibodies specific for the carboxyl terminus of Aβ40 were raised against a peptide corresponding to Aβ residues 36–40 (VGGVV). Monoclonal antibody JRF/cAβ40/10 recognized Aβ1–40 by ELISA and Western blot analysis, failed to detect Aβ1–42 by either of these methods, and did not detect full-length APP by immunoprecipitation or Western blot analysis. A similar Aβ42 carboxyl-terminal-specific antibody (JRF/cAβ42/26) was also generated. Additionally, an antibody was raised against a peptide corresponding to murine Aβ residues 1–15 (DAEFGHDSGFEVRHQ; antibody JRF/rAβ/2). This antibody detected murine but not human Aβ1–40, or Aβ1–42 by ELISA and Western blot analysis and was coupled to horseradish peroxidase for detection. Sandwich ELISAs using these antibodies were done as reported previously (30).

RESULTS

Expression of CD-MPR in Sporadic AD—We extended our earlier finding that CD-MPR immunoreactivity is increased in neurons in sporadic AD (9) by determining CD-MPR levels in human control and AD brain using Western blot analysis (Fig. 1). Prefrontal cortex obtained from age-matched control cases lacking tangle or plaque pathology was compared with the corresponding region from early stage AD cases, in which AD neuropathology was limited to the transentorhinal cortex/hippocampus, and from late stage AD cases as defined by the presence of severe neuronal loss and neurofibrillary and plaque pathology within the five surveyed brain regions (31–33). Quantitation of these results showed a mean increase of 3-fold in the immunosignal for CD-MPR in early stage AD cases (mean increase ± S.E., 3.1 ± 0.5; $p < 0.01$). CD-MPR levels in late stage AD cases increased to a smaller extent (1.5 ± 0.3; $p = 0.06$) However, as CD-MPR expression in the brain is primarily neuronal and is increased in pyramidal neurons in AD (9), the smaller increase in cases with advanced disease pathology compared with those with less severe disease is likely to be related to the extensive neurodegeneration and neuronal cell loss in late stage AD. Our results confirm that CD-MPR expression is substantially increased early in AD, as is the case for other AD-related abnormalities of the neuronal endosomal system (9).

Expression of CD-MPR Constructs in L Cells—To determine whether increased expression of the CD-MPR in AD might modify intracellular lysosomal hydrolase distribution and therefore the proteolytic processing of APP, we isolated a cDNA encoding the human CD-MPR and expressed this in murine L cells overexpressing human APP_{695} (Fig. 2). Extensive previous work has documented that CD-MPR is distributed, in order of decreasing abundance, within the trans-Golgi network, in endosomal compartments, and on the cell surface (16, 17, 23, 25, 26, 39, 40). The vesicular immunolabeling pattern for the transfected human CD-MPR, showing perinuclear as well as more peripheral labeling (Fig. 2*B*), is consistent with this distribution. Although we were unable to compare the level of

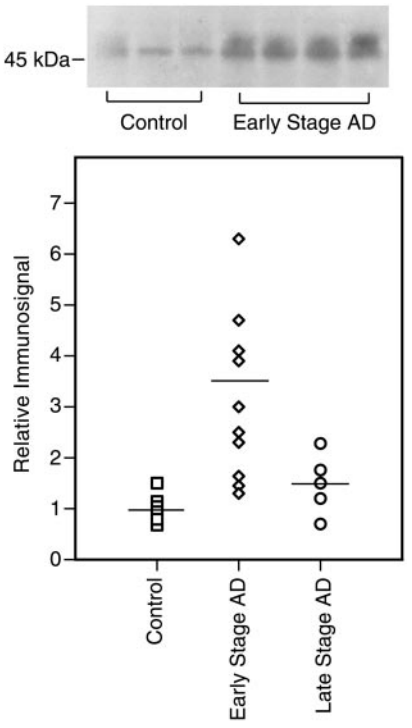

FIG. 1. **Expression of CD-MPR in control and AD brain.** Triton X-100-extracted proteins from human prefrontal cortex were sized by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with the anti-CD-MPR monoclonal antibody 22D4 (24) prior to quantitation. Normalized mean values ± S.E. are as follows: control, 1.0 ± 0.1, $n = 7$; early stage AD, 3.1 ± 0.5, $n = 10$ ($p < 0.01$); late stage AD, 1.5 ± 0.3, $n = 5$ ($p = 0.06$). The *top panel* is a representative Western blot showing three control and four early stage AD cases.

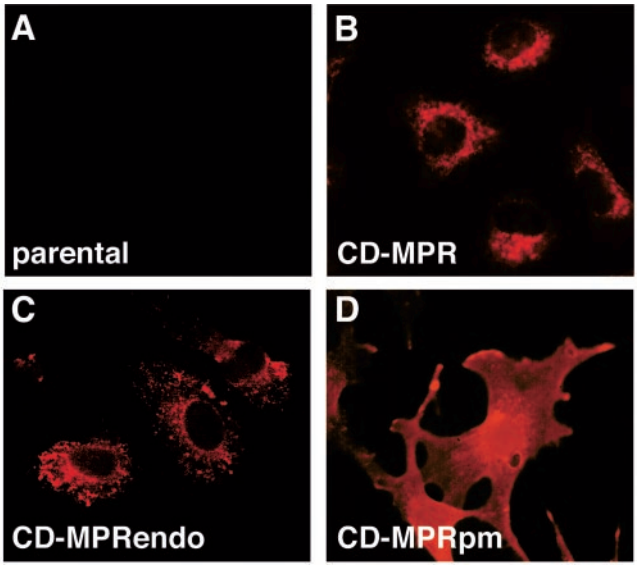

FIG. 2. **Expression of human CD-MPR and CD-MPR constructs in L cells.** The L cell line overexpressing APP (*A*) and this line stably transfected with cDNAs encoding human CD-MPR (*B*), CD-MPRendo (*C*), and CD-MPRpm (*D*) were fixed, permeabilized, and immunolabeled with a monoclonal antibody specific for the luminal/ectodomain of human CD-MPR (23).

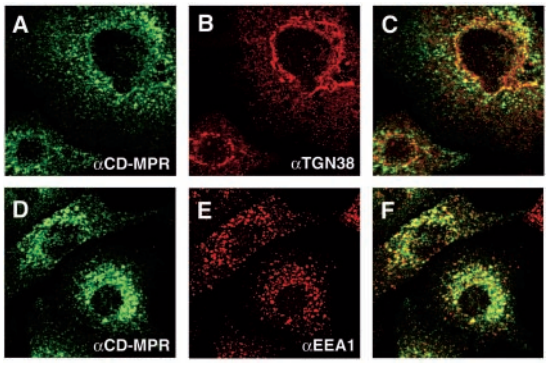

FIG. 3. **CD-MPR colocalizes with TGN and early endosomal markers.** CD-MPR-expressing L cells were imaged by confocal microscopy following double immunolabeling with the monoclonal antibody directed against human CD-MPR (*A* and *D*) and polyclonal antibodies that recognize TGN38 (*B*) or EEA1 (*E*). *Panels C* and *F* show the coincidence of CD-MPR with both of these markers (*yellow-orange color*).

expression of the human CD-MPR directly with that of the endogenous mouse CD-MPR in this cell line, because of the species specificity of our antibodies, we did compare the expression of human CD-MPR in the transfected L cells to its expression in control and AD brain. When standardized to equal amounts of total protein examined by Western blot analysis, the expression of CD-MPR in L cells following induction was ~2-fold that seen in early stage AD brain and ~5.5-fold that seen in controls (data not shown). Given the cellular heterogeneity of the brain and the predominant localization of CD-MPR to neurons, it is likely that the expression in neurons in AD brain is close to, if not higher than, that in CD-MPR transfected L cells. This suggests that although the overexpression of CD-MPR in the L cell line may not match precisely the level in AD brain, it is likely to be similar, allowing trends in APP processing brought about by an increase in CD-MPR expression to be examined in this model.

Although it is the increased expression of CD-MPR that has relevance to AD, we also designed and expressed two trafficking mutants of CD-MPR as controls. These trafficking mutants have modified cytoplasmic domains but intact mannose 6-phosphate hydrolase binding luminal/extracellular domains. Both constructs, because of their differing localization in the cell, offer additional information as to the importance of a particular compartment in APP processing. In the CD-MPRendo construct, the cytoplasmic tail of the CD-MPR was removed and replaced by a well characterized 10-amino acid sequence containing a tyrosine-endocytosis motif (the cytoplasmic tail of influenza hemagglutinin (HA) with the C543Y substitution (41)). Consistent with the early endosomal distribution previously reported by Roth and colleagues (41, 42) for the HA C543Y mutant, immunolabeling of the CD-MPRendo construct showed a punctate pattern distributed throughout the cell body (Fig. 2*C*), which colocalized with early endosomal markers (data not shown). The other CD-MPR trafficking mutant, CD-MPRpm, contains the wild-type HA cytoplasmic tail, which is responsible for the exclusion of HA from clathrin-coated pits and therefore promotes its accumulation in the plasma membrane (43). As expected, immunolabeling of CD-MPRpm showed a typical cell surface pattern (Fig. 2*D*), which was similar in both nonpermeabilized (shown) and permeabilized cells (not shown).

Fig. 3 shows confocal immunolocalization of the overexpressed human CD-MPR with a marker of the trans-Golgi network (TNG38) (29, 44) as well as a marker of early endosomes, EEA1 (27, 45). As expected, a significant overlap of the overexpressed CD-MPR and TNG38 was seen (Fig. 3, *A–C*). However, much of the abundant, punctate labeling seen more peripherally in the cell, which did not colocalize with TGN38, was found to colocalize with EEA1 (*panels D–F*). The fact that significant CD-MPR immunolabeling can be detected in early endocytic compartments could underlie a partial redistribution of CD-MPR ligands to the early endosome observed in AD (9).

Localization of Cathepsin D, a Marker Lysosomal Protease, in CD-MPR-overexpressing Cells—To demonstrate whether overexpression of CD-MPR resulted in a partial redistribution of lysosomal hydrolases, we characterized the intracellular distribution of cathepsin D, a protease delivered to lysosomes by both the CD-MPR and the CI-MPR (46). In L cells not expressing a human CD-MPR construct, Cat D immunolabeling revealed a typical lysosomal pattern (Fig. 4*A*). However, in L cells overexpressing CD-MPR, significant Cat D immunolabeling was also seen in smaller punctate compartments (Fig. 4*B*). A similar pattern of small Cat D immunopositive compartments was seen in CD-MPRendo-expressing cells (Fig. 4*C*), suggesting that expression of these constructs increased Cat D levels in early endosomal compartments. In CD-MPRpm-expressing cells, some cell depletion of intracellular Cat D signal was occasionally evident, although the more typical pattern of Cat D immunolabeling in CD-MPR cells was similar to that seen in control cells (Fig. 4*D*).

We examined the distribution of Cat D in CD-MPR cells compared with control L cells by subcellular fractionation over a density gradient (Fig. 4*E*). In the control cells, the bulk of Cat D (both mature and heavy chain) was found in the more dense fractions (fractions 3–5) as would be expected for a lysosomal hydrolase. In cells overexpressing CD-MPR, a greater proportion of the Cat D immunosignal was seen in the lightest fractions (fractions 1 and 2), whereas the denser fractions showed a relative reduction in Cat D levels, particularly the proteolytically processed heavy chain. This finding argues that increased expression of the CD-MPR can affect the distribution of a marker lysosomal hydrolase, partially shifting the enzyme from the dense lysosomal fractions to lighter endosomal fractions.

To further demonstrate that Cat D is partially redistributed to early endocytic compartments, we incubated living cells in fluorescently tagged pepstatin A (BODIPY-pepstatin A, which binds with high affinity to active Cat D (47)). It has been shown previously that Cat D can be activated within endocytic compartments and that active forms of the enzyme can be found there (48). Thus, we hypothesized that in cells incubated in BODIPY-pepstatin A, binding would be more rapid in cells having higher levels of active Cat D in early endosomes. With longer incubation, however, the bulk of the Cat D in lysosomes would also label and become the predominant BODIPY-pepstatin A signal. As expected, L cells incubated for 10 min in BODIPY-pepstatin A showed no fluorescence (Fig. 5*A*). In contrast, CD-MPR-overexpressing cells, following a 10-min incubation, showed BODIPY-pepstatin A labeling of small vesicles, consistent in distribution and size with early endosomes (Fig. 5*B*). Cells expressing CD-MPRendo showed a similar fluorescence pattern, although typically with more robust labeling (Fig. 5*C*). In both cases, the addition of excess unlabeled pepstatin eliminated labeling, arguing that the uptake of BODIPY-pepstatin in these cells could be competed and was therefore specific (data not shown). CD-MPRpm-expressing cells did not rapidly take up BODIPY-pepstatin (Fig. 5*D*). We found that differences among the four cell lines were much less apparent after longer incubation (30 min), at which time each line showed significant BODIPY-pepstatin labeling (data not shown). After a 2-h incubation in BODIPY-pepstatin A, the

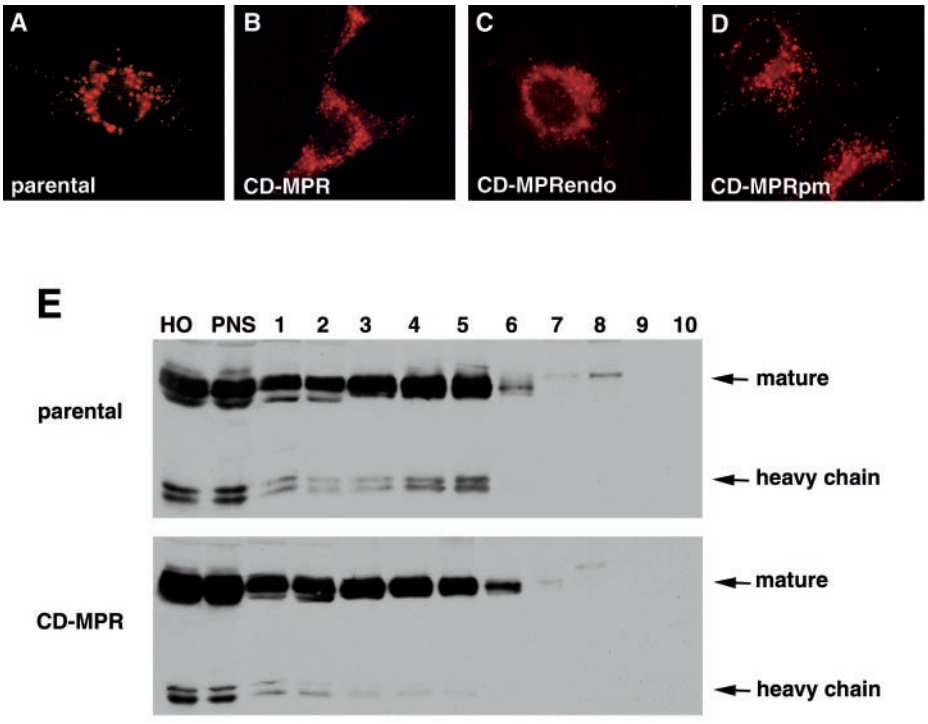

FIG. 4. **Expression of human CD-MPR in L cells partially redistributes cathepsin D.** The *top panels* show immunolabeling for Cat D in control L cells (*A*) as well as in CD-MPR (*B*)-, CD-MPRendo (*C*)-, and CD-MPRpm (*D*)-expressing cells. In *E*, cell homogenates were prepared and fractionated by ultracentrifugation over an Optiprep gradient as described under "Materials and Methods." Western blot analysis was performed to show the distribution of Cat D within each fraction (*1*, lightest, to *10*, most dense) as well as the starting homogenate (*HO*) and the post-nuclear supernatant (*PNS*) that were loaded onto the gradient. The *mature* and *heavy chain* of Cat D are indicated.

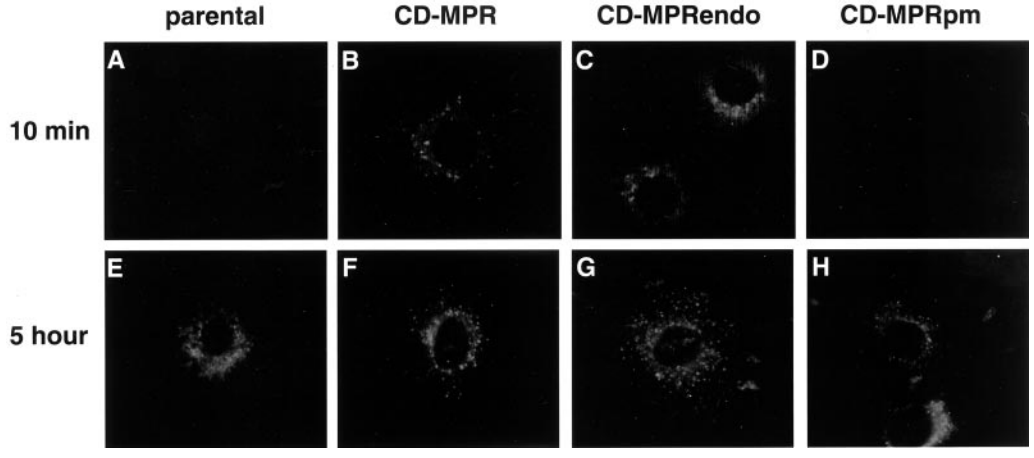

FIG. 5. **Active Cat D can be detected in early endosomal compartments in the CD-MPR line.** *A–D*, living cells were incubated in fluorescently tagged pepstatin A (BODIPY-pepstatin A) for 10 min, which binds with high affinity to active cathepsin D (47). *Panels E–H* show BODIPY-pepstatin labeling after a 2-h incubation in the same cell lines.

expected lysosomal pattern was seen in every cell line (Fig. 5, *E–H*). These data are consistent with a partial redistribution of lysosomal hydrolases within the endosomal-lysosomal system to early endosomes in a CD-MPR-overexpressing cell.

Expression of CD-MPR Constructs Does Not Alter APP Bulk Degradation or Processing to sAPP—Although all CD-MPR constructs were transfected into the same clonal APP-overexpressing cell line, we confirmed equal levels of APP expression in each line by metabolic labeling (Fig. 6) and by Western blot analysis (Fig. 7). Pulse labeling of equal numbers of cells followed by immunoprecipitation with a monoclonal antibody directed against the carboxyl terminus of APP (C1/6.1) showed equal levels of APP biosynthesis in the parental APP-overexpressing cell line as well as in each of the CD-MPR construct co-transfectants (Fig. 6*A*, *lanes 1*, *5, 9*, and *13*). In addition to expressing comparable levels of APP, these lines showed similar rates of APP turnover. The majority of cellular APP detected with C1/6.1 was degraded within a 2-h chase period (*lanes 2*, *6, 10*, and *14*), as is the case for endogenous APP in L cells (not shown) and as has been reported in other cell lines (49, 50). We also examined the secretion of soluble APP fragments (sAPP) into the growth media by subjecting conditioned media to sequential immunoprecipitation with antibody 6E10, which detects sAPP cleaved at the α-cleavage site but not β-cleaved sAPP, followed by an antibody that recognizes all sAPP species (P2-1) (35, 49, 51). The depletion of sAPPα by 6E10 was confirmed in parallel experiments in which little sAPP was recovered by subsequent 6E10 immunoprecipitations of the same sample. In the experiment shown in Fig. 6*A*, metabolically labeled sAPPα and sAPPβ were immunoprecipitated from each cell line following a 2-h chase. Again, levels of sAPPα and sAPPβ in the chase media were similar among these cell lines. It is also noteworthy that nearly one-quarter of the soluble APP released from these cells lacks the 6E10 epitope and is therefore presumed to be cleaved at or near the β-cleavage site (compare *lanes 3* and *4*, *7* and *8*, *11* and *12*, and *15* and *16* in Fig. 6*A*).

As the CD-MPR-overexpressing line is the model relevant to early changes in sporadic AD, we compared APP metabolism in this line to the parental APP-overexpressing cell line with finer

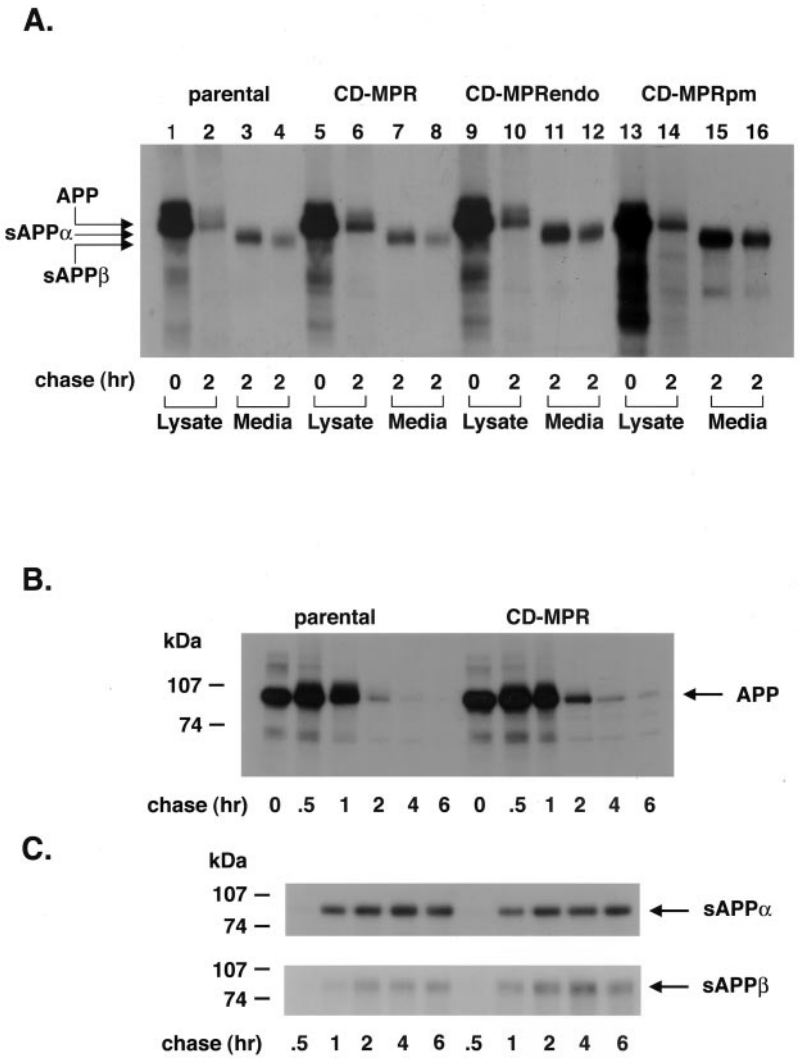

FIG. 6. **Secreted APP is unaffected by CD-MPR overexpression and expression of CD-MPR trafficking mutants.** Cells were labeled metabolically for 15 min and chased for the indicated times. Full-length APP was immunoprecipitated from cell lysates and secretory APP from the growth media as indicated. Monoclonal antibody C1/6.1 was used to immunoprecipitate full-length APP from cell lysates. This antibody recognizes both the overexpressed human APP as well as the endogenous mouse APP. sAPP was detected in the media using sequential immunoprecipitations with antibodies 6E10 and P2-1 to differentiate between sAPPα and sAPPβ as described previously (35, 49, 51).

time resolution (Fig. 6*B*). Again, cells were pulse/chase-labeled, and either cell lysates or media were subjected to immunoprecipitation. In both the parental and CD-MPR-overexpressing lines, intracellular turnover of APP was rapid, with most labeled full-length APP lost between 1 and 2 h of chase. Both sAPPα and sAPPβ were first detected in the growth media of both lines after a 1-h chase (Fig. 6*C*). Consistent with the disappearance of cellular full-length APP by 2-h chase time, levels of sAPPα and sAPPβ in the media peaked at 2 h of chase time and were found to remain relatively stable through a 6-h chase period. These results indicate that the bulk metabolism of APP is not substantially changed by the expression of any of the CD-MPR constructs.

Increased Aβ Secretion in CD-MPR and CD-MPRendo—In each of these cell lines, we determined the amount of human Aβ40 and Aβ42 secreted into the growth media by sandwich ELISA. Western blot analysis (Fig. 7*A*) demonstrated comparable levels of APP expression in each cell line. Cells overexpressing CD-MPR showed a 2.6-fold increase in Aβ40 and Aβ42 secreted into the growth media during an 8-h incubation (Fig. 7*B*). Similar increases were seen in cells expressing the control construct CD-MPRendo (2.6-fold increase in Aβ40 and 2.8-fold increase in Aβ42), whereas expression of the CD-MPRpm construct had little effect on Aβ. In these cell lines, the ratio of Aβ40/Aβ42 remained unchanged.

We confirmed this finding in a second series of L cells expressing the CD-MPR constructs but not overexpressing APP. Using a sensitive sandwich ELISA that employs carboxyl-terminal Aβ40- or Aβ42-specific capture monoclonal antibodies and a murine Aβ-specific detection monoclonal antibody, we were able to detect endogenous murine Aβ40 secreted from L cells, although Aβ42 remained below detection levels (Table I). As seen in the APP-overexpressing cells, CD-MPR and CD-MPRendo expression increased Aβ40 secretion into the growth media by nearly 3-fold.

DISCUSSION

In this study, we modeled the alterations in hydrolase trafficking we had observed in sporadic AD brain by reproducing in a cell line the increased CD-MPR expression seen in the disease (9). Our results show that increased expression of CD-MPR leads to an increased production of Aβ40 and Aβ42 and that a likely mechanism is increased trafficking of lysosomal hydrolases to early endosomes. The findings of this study are in agreement with several previous *in vitro* studies showing that the endocytic pathway is a major site of APP processing leading to Aβ generation (3, 4, 52) and suggest that early endosomes may function as an important compartment for enhanced Aβ generation and release in sporadic AD.

Mechanisms of Increased Aβ Secretion—A number of cellular mechanisms could contribute to the increase in Aβ levels measured in the media following CD-MPR overexpression. First, CD-MPR is important for the delivery of many lysosomal hydrolases to compartments within the endosomal-lysosomal system. Overexpression of CD-MPR partially redistributes these enzymes to early endosomes where they may interact with APP

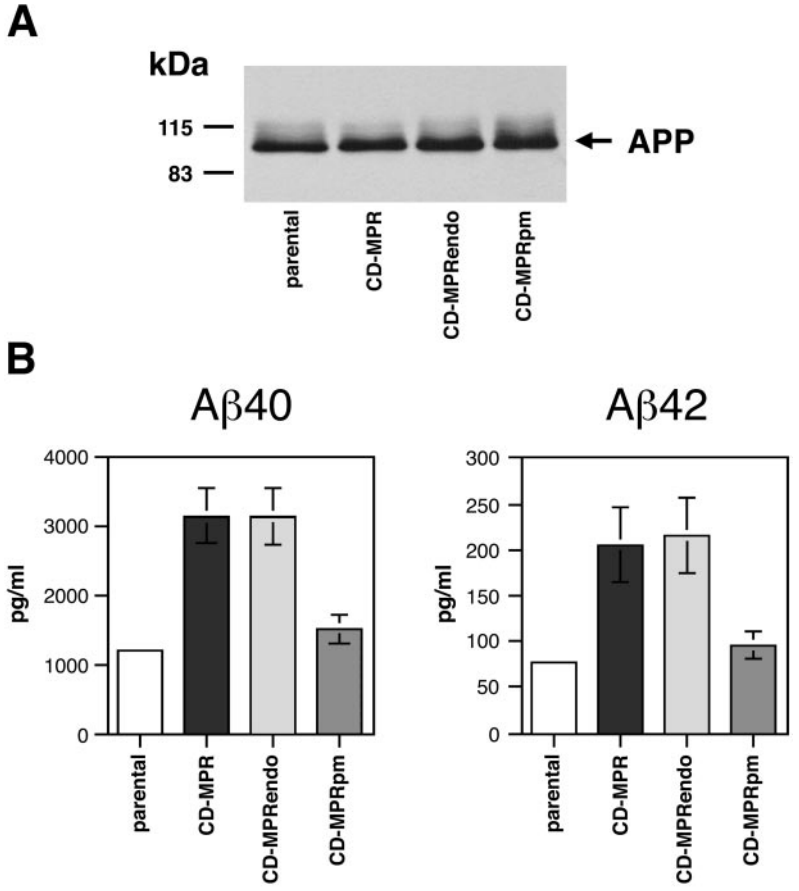

FIG. 7. **CD-MPR overexpression and CD-MPRendo expression result in increased Aβ secretion into the growth media.** *A*, the expression of full-length APP was examined by Western blot analysis using the monoclonal antibody C1/6.1. Levels of APP expression were similar in lysates prepared from all four L cell lines. Secretion into the growth media of Aβ40 and Aβ42 by each line was measured using a sandwich ELISA (*B*) (37). The results shown are the mean ± S.E. of four experiments and are normalized to total cellular protein; the increased Aβ40 and Aβ42 secreted by CD-MPR- and CD-MPRendo-expressing cells compared with the parental APP-overexpressing line is statistically significant ($p < 0.01$).

TABLE I
Murine Aβ40 secretion is increased by CD-MPR overexpression and CD-MPR expression

The CD-MPR constructs were stably expressed in L cells not overexpressing human APP, and levels of Aβ40 secreted into the growth media were determined by sandwich ELISA using a murine Aβ-specific detection monoclonal antibody (see "Materials and Methods"). Aβ42 levels were below the detection limit. A representative experiment of three is shown.

Cell line	Murine Aβ40
	pg/ml ± S.E.
L cell	23.3 ± 0.4
CD-MPR	69.4 ± 3.4 ($p < 0.05$)
CD-MPRendo	64.4 ± 6.8 ($p < 0.05$)
CD-MPRpm	41.1 ± 4.7

leading to Aβ generation and secretion. Although our data do not address the identity of specific lysosomal hydrolases(s) that may be involved in APP processing, one candidate is the aspartyl protease Cat D, which is trafficked by the mannose 6-phosphate motif (46), has β-secretase activity in some systems (8, 53–55), and has a genetic polymorphism that has been linked in some studies to increased risk for AD (56, 57), although not all studies have found this linkage (58–61). Our previous studies have shown that the neuronal expression, trafficking, and intracellular distribution of Cat D are significantly abnormal in AD (9, 11, 12), although these studies typically employed Cat D as a marker for more generalized changes in lysosomal hydrolase expression and intracellular distribution, as did the current study. Saftig and colleagues (62) have shown that Aβ is secreted from hippocampal neurons isolated from Cat D knockout mice, indicating that there is not a requirement for Cat D in the basal and nonpathological generation of Aβ. In AD, however, increased expression and mistrafficking of Cat D or another lysosomal hydrolase(s) with potential secretase activity to early endosomes, a site where APP normally resides, remains an appealing mechanism for linking endocytic pathway abnormalities with β-amyloidogenesis (10).

A second possible mechanism does not involve a CD-MPR-trafficked protease directly in APP cleavage. Instead, one or more such proteases could be involved in the regulation of an APP secretase. For example, although the delivery of the BACE family of β-secretases to the endosomal-lysosomal system is unlikely to depend upon a mannose 6-phosphate signal, it has recently been shown that a propeptide sequence is removed from BACE (6). Although BACE activation appears to occur in the secretory pathway, activation could also occur in an endosomal compartment. Changing the activity of lysosomal proteases within early endosomes could potentially modulate BACE activity in endocytic compartments.

Regardless of the proteases involved, CD-MPR overexpression did not change the ratio of Aβ40 to Aβ42, a result that would most readily be explained by increased β-secretase activity. Nevertheless, we did not detect large changes in the secretion of sAPPα or sAPPβ (Fig. 6). This finding contrasts with what has been seen with the Swedish mutation of APP, which enhances β-cleavage in the Golgi apparatus to such an extent that sAPPβ secretion increases dramatically, as does the intracellular accumulation of β-cleaved carboxyl-terminal APP stubs that are thought to act as a "precursor pool" for subsequent γ-cleavage and Aβ release (49, 50). Because of the rapid recycling of endosomal contents to the cell surface, relatively subtle changes of APP proteolysis within the early endosome may contribute substantially to Aβ release from the cell. Likewise, the rapid delivery of early endosomal membrane to the lysosome may prevent the accumulation of

APP carboxyl-terminal fragments, which have been shown to be degraded within the lysosome (63, 64). Perhaps most significantly, the lack of detectable change in sAPPα and sAPPβ levels while Aβ levels undergo a substantial change likely reflects the fact that only a very small percentage of APP is processed into Aβ compared with that cleaved to sAPP or completely degraded.

Relationship to AD Pathobiology—The presenilin mutations and many of the APP mutations that cause early onset familial AD increase either total Aβ production and/or production of long forms of Aβ (Aβ42/43), thereby tipping the scales in favor of Aβ accumulation. Although an increase in Aβ production has not yet been documented in sporadic AD, the increase in Aβ secretion seen in our CD-MPR model of sporadic AD suggests one potential mechanism. It is worth noting that a model using CD-MPR overexpression only partially reproduces the spectrum of changes seen in early endosomes during AD. The increased expression in sporadic AD of Rab5, Rab4, rabaptin, and EEA1, all involved in the regulation of endocytosis or recycling, suggests that endocytosis itself is up-regulated in the disease (9, 10). Moreover, the up-regulation of the early endosomal system seen in sporadic AD is affected by the Apo E genotype, with individuals who carry one or two copies of Apo E_4 showing the greatest increase in early endosome size (10). Given the importance of the endocytic pathway in Aβ generation, an increase in endocytosis could have the effect of increasing the internalization of APP from the cell surface and thereby further enhancing endosomal Aβ generation. The results of this study suggest that dysfunction of the endocytic pathway can lead to an increase in Aβ secretion and, therefore, in sporadic AD could shift the balance toward Aβ accumulation. Although CD-MPR overexpression is likely to only partially, and imperfectly, model AD-related changes of the endosomal system, to our knowledge it is the only model in which AD-related cellular changes known to occur in sporadic forms of the disease lead to increased Aβ generation.

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