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Rab5-stimulated Up-regulation of the Endocytic Pathway Increases Intracellular β-Cleaved Amyloid Precursor Protein Carboxyl-terminal Fragment Levels and Aβ Production*

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Olivera M. Grbovic‡§¶, Paul M. Mathews‡§‖, Ying Jiang‡, Stephen D. Schmidt‡, Ravi Dinakar‡, Nicole B. Summers-Terio‡, Brian P. Ceresa, Ralph A. Nixon‡‖‡‡, and Anne M. Cataldo‡§§**

*From the ‡Center for Dementia Research, Nathan Kline Institute, Orangeburg, New York 10962, the Departments of ‖Psychiatry and ‡‡Cell Biology, New York University School of Medicine, New York, New York 10016, the **Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, and the §§Laboratory for Molecular Neuropathology, Mailman Research Center, McLean Hospital, Belmont, Massachusetts 02478 and the Department of Psychiatry and Neuropathology, Harvard Medical School, Boston, Massachusetts 02115*

We previously identified abnormalities of the endocytic pathway in neurons as the earliest known pathology in sporadic Alzheimer's disease (AD) and Down's syndrome brain. In this study, we modeled aspects of these AD-related endocytic changes in murine L cells by overexpressing Rab5, a positive regulator of endocytosis. Rab5-transfected cells exhibited abnormally large endosomes immunoreactive for Rab5 and early endosomal antigen 1, resembling the endosome morphology seen in affected neurons from AD brain. The levels of both Aβ40 and Aβ42 in conditioned medium were increased more than 2.5-fold following Rab5 overexpression. In Rab5 overexpressing cells, the levels of β-cleaved amyloid precursor protein (APP) carboxyl-terminal fragments (βCTF), the rate-limiting proteolytic intermediate in Aβ generation, were increased up to 2-fold relative to APP holoprotein levels. An increase in β-cleaved soluble APP relative to α-cleaved soluble APP was also observed following Rab5 overexpression. βCTFs were co-localized by immunolabeling to vesicular compartments, including the early endosome and the trans-Golgi network. These results demonstrate a relationship between endosomal pathway activity, βCTF generation, and Aβ production. Our findings in this model system suggest that the endosomal pathology seen at the earliest stage of sporadic AD may contribute to APP proteolysis along a β-amyloidogenic pathway.

In the brain parenchyma, the deposition of the small peptide Aβ, a proteolyic fragment of the amyloid precursor protein (APP),[1] is an invariant feature of Alzheimer's disease (AD) (1). Generation of Aβ requires two sequential proteolytic steps: β-cleavage in the lumenal domain of APP followed by intramembranous γ-cleavage (reviewed in Ref. 2). β-Cleavage appears to be mediated by the BACE family of aspartyl-proteases (3, 4), whereas γ-cleavage requires the presenilin protein complex (5, 6). The intracellular site(s) of Aβ generation has been the subject of intense investigation for a number of years, and both the secretory and endocytic pathways have been implicated (7–13). Confounding the clear delineation of Aβ generation sites is the broad intracellular distribution of the key proteins involved. APP is predominantly localized to the trans-Golgi network (TGN), but significant amounts of the protein are found at the cell surface and within endosomes and other transport vesicles (2). BACE has been placed within early endosomes and/or throughout the endosomal-lysosomal system (3, 14, 15), although a predominant TGN localization has also been reported (16, 17). The presenilin proteins were initially thought to be located primarily within the endoplasmic reticulum and Golgi apparatus (18–23), but more recent evidence suggests a distribution also within the plasma membrane and endosomes (18, 24–27), and studies of nicastrin also strongly suggest that the mature presenilin complex is found in compartments distal to the endoplasmic reticulum (28).

Our previous studies have shown that abnormalities of the neuronal endocytic pathway precede substantial β-amyloid deposition in AD brain and appear decades prior to the development of amyloid pathology in Down's syndrome (29). These abnormalities include increased early endosome volume, increased expression or altered localization of proteins involved in the regulation of endocytosis, early endosomal fusion, and recycling (such as Rab5, early endosomal antigen 1, rabaptin 5, and Rab4), and an atypical increase in the levels of lysosomal hydrolases within the early endosome (29, 30). Based on these observations and the known role of the endocytic pathway in APP metabolism (7–9), we have postulated that the early endosomes abnormalities seen in sporadic AD may be directly related to a rise in Aβ levels and subsequent β-amyloid accumulation. Recently, we modeled one aspect of these endocytic pathway changes, the increased expression of the 46-kDa mannose-6-phosphate receptor, which can mediate enhanced delivery of lysosomal proteases to early endosomes, and showed that this led to increased Aβ secretion from cells (31).

In this study we have modeled a second aspect of AD-related endocytic pathway abnormalities, such as the apparent increase in endocytosis and early endosomal fusion seen in sporadic forms of the disease, by overexpressing Rab5 in cells. Rab5 is a member of the Ras family of small GTPases that are

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§ These authors contributed equally to this study.

¶ To whom correspondence should be addressed: Memorial Sloan-Kettering Cancer Center, Dept. for Cell Biology, R1121, 1275 York Ave., New York, NY 10021. E-mail: grbovico@mskcc.org.

[1] The abbreviations used are: APP, amyloid precursor protein; AD, Alzheimer's disease; TGN, trans-Golgi network; EEA1, early endosomal antigen 1; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; GST, glutathione *S*-transferase; ApoE, apolipoprotein E; mAb, monoclonal antibody.

critical modulators of vesicular transport in cells (32–34). In addition to its association with early endosomes, Rab5 is localized to the plasma membrane, clathrin-coated pits, and recycling endosomes. Along with the effector proteins rabaptin 5 (35–37) and early endosomal antigen 1 (EEA1) (38–40), Rab5 plays a key role in modulating early endosomal fusion and the membrane docking events involved in endocytosis and recycling (34, 41). Here, we show that Rab5 overexpression reproduced many of the abnormal morphological features of early endosomes seen *in vivo* in neurons from AD and Down's syndrome brain. Significantly, overexpression of Rab5 dramatically increased both the levels of secreted Aβ40 and Aβ42. Cells overexpressing Rab5 also had elevated levels of the β-cleaved APP carboxyl-terminal fragment (βCTF), which partially colocalized to early endosomal compartments.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfections—Murine fibroblast-like L cells stably transfected with human APP_{695} have been described (L/APP cells) (31, 42). A full-length human Rab5 cDNA was isolated and subcloned into the expression vector pcDNA3. Semiconfluent cells grown in a 35-mm-diameter culture dish were transfected with 0.5–3 μg of Rab5 plasmid cDNA/5 × 10^5 cells using FuGENE 6 as described by the manufacturer's protocol (Roche Applied Science). Throughout, the expression of APP and Rab5 was induced by incubating cells for ~40 h in medium containing 20 mM sodium butyrate (31).

Antibodies—An affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 193–211 of human Rab5 (Santa Cruz Biotechnology, Santa Cruz, CA) and a second human-specific monoclonal antibody generated against the Rab5 GTP binding motifs II and III (BD Biosciences/Transduction Laboratories) were used. Purified EEA1 rabbit serum was the kind gift of Dr. S. Corvera of the Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA) (38, 43). The key anti-APP/APP metabolite antibodies used in this study are described in Table I. C1/6.1 (42) and C2/7.1 recognize distinct epitopes within the carboxyl terminus of APP. The anti-APP-lumenal domain monoclonal antibody P2-1 was a generous gift from Dr. Maria Kounnas at Sibia/Merck (72); monoclonal antibodies 6E10 and 4G8 were obtained commercially (Signet Laboratories Inc., Dedham, MA). Monoclonal antibody JRF/AβN/25, which recognized the βCTF and Aβ uniquely, has been previously characterized in detail (42, 44).

Immunolabeling—Cells grown on glass coverslips were gently fixed in 4% formaldehyde, 5% sucrose in phosphate-buffered saline, pH 7.4, on ice for 30 min, washed with phosphate-buffered saline, and immunolabeled as previously described (31). The cells were permeabilized by the addition of 0.1% saponin or 0.3% Triton X-100 (Sigma) to the antibody-containing solutions. The coverslips were mounted in GelMount (Fisher) and viewed using epifluorescent or laser confocal scanning microscopy.

Receptor-mediated and Fluid Phase Uptake—Receptor-mediated uptake was examined using fluorescent-tagged transferring Tf-Alexa 586 (Molecular Probes, Eugene, OR). The cells grown on glass coverslips were washed and incubated for 30 min at 37 °C in phosphate-buffered saline containing 0.1% bovine serum albumin and 20 mM HEPES. The cells were then incubated with transferring (10 μg/ml) on ice for 1 h and washed three times with phosphate-buffered saline with bovine serum albumin prior to being warmed to 37 °C for 0, 3, 5, 10, and 15 min. Following fixation, the cells were immunolabeled with anti-Rab5 antibody.

Fluid phase uptake was evaluated in cells that were incubated for 0, 3, 5, 10, and 15 min at 37 °C with prewarmed serum-free medium containing 5 mg/ml horseradish peroxidase (HRP) (HRP type II; Sigma). The cells were washed and fixed with 4% paraformaldehyde, 0.2% gluteraldehyde in 0.1 M cacodylate buffer. Ultrathin sections cut from Epon-embedded cells were placed on 300 mesh, uncoated grids, lightly post-stained in uranyl acetate and lead citrate and examined using a JEOL 1200EX electron microscope (45).

Metabolic Labeling and Immunoprecipitation—5 × 10^5 cells were seeded onto 35-mm culture dishes and transfected with 1 μg of Rab5 cDNA. Following induction for ~40 h, the cells were metabolically labeled using Tran^{35}S-label (PerkinElmer Life Sciences), and the cell lysates were prepared in 1% Triton X-100 immunoprecipitated as previously described (42). sAPPβ and sAPPα were isolated from conditioned medium using sequential immunoprecipitation with 6E10 and P2-1 as previously characterized (10, 31). Labeled Aβ from conditioned medium or cell lysates was immunoprecipitated using 4G8 and 6E10. Immunoprecipitated proteins were sized by SDS-PAGE, and labeled proteins were visualized by exposure to x-ray film and analyzed using a Storm 840 PhosphorImager (Molecular Dynamics) and/or by densitometric scan analysis (NIH Image).

Western Blot Analysis—Equal amounts of total proteins were separated on SDS-PAGE, transferred to polyvinylidene difluoride membranes, incubated in primary antibody overnight, washed, and incubated with HRP-conjugated secondary antibody (31). Membranes were incubated in ECL substrate (Amersham Biosciences) and exposed to x-ray film.

ELISAs—5 × 10^5 cells were seeded onto 35-mm culture dishes and transfected as described above. The conditioned medium was collected after 40 h, and debris was removed with a rapid centrifugation and frozen in aliquots prior to ELISA determination of Aβ levels. Following the collection of conditioned medium, Triton X-100 lysates (1% Triton X-100, 140 mM NaC1, 25 mM Tris, pH 7.4, 0.5 mM EDTA, 1 mM EGTA, and protease inhibitors) were prepared and frozen for later determination of cell-associated APP metabolite levels. The Aβ40- and Aβ42-specific sandwich ELISAs used by our laboratory and the determination of human Aβ levels in the conditioned medium of cells have been described in detail (42).

Cell-associated βCTF levels were determined using a sandwich ELISA employing C1/6.1 as the capture antibody and JRF/AβN/25 as the detection antibody. The cell lysates were loaded neat or diluted 1:2 into Nunc-Immuno Plates (Nunc A/S, Roskilde, Denmark) previously coated with 10 μg/ml C1/6.1 in 10 mM Tris, 10 mM NaCl, 0.05% NaN_3, pH 8.5, and blocked as described for the Aβ ELISAs (42). βCTFs were detected by incubating for 4 h at room temperature with HRP-conjugated JRF/AβN/25 followed by a colorimetric assay (42). A synthetic peptide (**DAEFRHD***KMQQNGYENPTYKFFEQMQN*), kindly provided by Dr. D. Westaway at the University of Toronto, containing the JRF/AβN/25 epitope at its amino terminus (in bold type) and the C1/6.1 epitope at its carboxyl terminus (in italics) was used as a standard.

Using an aliquot of the Triton X-100 cell lysate, an additional ELISA developed to detect APP holoprotein as well as the cell-associated CTFs (APP/total CTF ELISA) was run. This ELISA uses C1/6.1 as the capture antibody, as does the βCTF ELISA, and employs a second carboxyl-terminal APP antibody for detection (C2/7.1; see Table I). A glutathione *S*-transferase fusion protein containing the carboxyl-terminal 99 residues of human APP and both the C1/6.1 and C2/7.1 epitopes (GST-βPP672–770) (46), a generous gift of Dr. E. Levy at New York University, was used as the standard in this ELISA. The βCTF ELISA and the APP/total CTF ELISA have the sensitivity to detect these metabolites in cell extracts (~8 and 250 fmol/ml cell lysate, respectively; see Fig. 6*A*) and show strong correlation with βCTF or APP holoprotein levels when analyzed by other methods (data not shown). Throughout, ELISA measurements were determined from standard curves run on the same plate and represent the means of two or more wells.

RESULTS

Endocytic Pathway Up-regulation by Overexpression of Rab5—Up-regulation of the endocytic pathway and its potential effect on APP processing was modeled in L cells stably overexpressing APP695 (L/APP) that were transiently transfected with human Rab5. Efficiency of transfection in multiple experiments was assessed by counting human Rab5-immunoreactive cells in randomly chosen fields, and typical levels of expression are shown in Fig. 1. The human-specific Rab5 mAb did not label nontransfected cells (Fig. 1*A*) but consistently labeled more than ~45% of the cells when transfected with 0.5 μg of Rab5 cDNA/5 × 10^5 cells. This percentage increased close to 75% as the amount of Rab5 cDNA was increased (~55% at 1.0 μg/5 × 10^5 cells and ~75% at 3.0 μg/5 × 10^5 cells). Western blot analysis showed that although Rab5 protein levels increased significantly with the cDNA amounts used for transfections, Rab5 protein levels increased considerably more than percentage of transfected cells (Fig. 1*B*). This observation reflected the finding that at the higher cDNA amounts individual Rab5 transfected cells expressed extremely high levels of protein, which was found by immunolabeling to fill the cytosol rather than increase the labeling of endosomes (Fig. 1*A*).

We next sought to correlate Rab5 overexpression in this

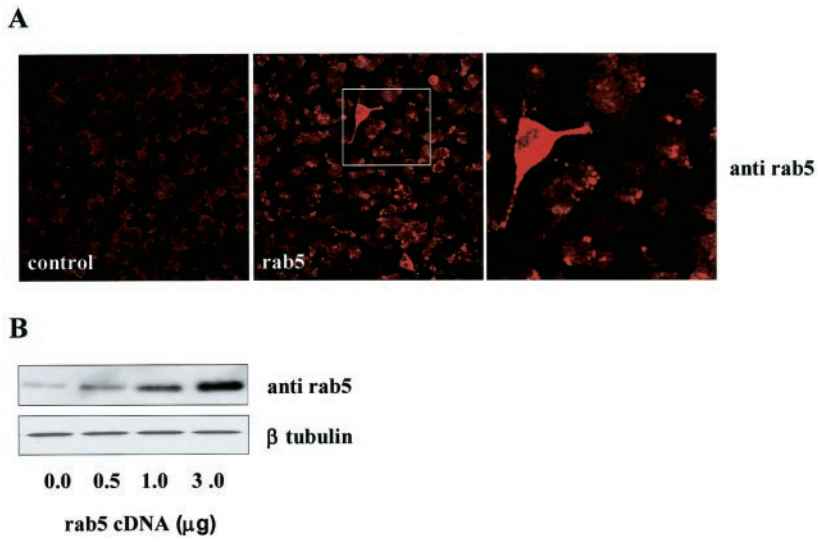

FIG. 1. **Transient transfection of L/APP cells with human Rab5 cDNA.** *A*, cells treated with FuGENE 6 reagent alone (control) and immunolabeled with anti-human Rab5 mAb showed only diffuse background labeling without specific endosomal labeling. When transfected with 1.0 μg of Rab5 cDNA/5 $\times$ 10^5 cell, ~55% of the cells, based on cell counts from multiple transfections, showed distinct and punctate human Rab5 labeling. Individual cells that showed the highest levels of human Rab5 expression contained Rab5 immunosignal throughout the cytosol (*right panel*). *B*, typical result of Western blot analysis with an anti-Rab5 polyclonal antibody that recognizes both the mouse and human proteins on cells transfected with increasing amounts of Rab5 DNA as indicated. Equivalent protein loading was confirmed in the same blot using an antibody against β-tubulin.

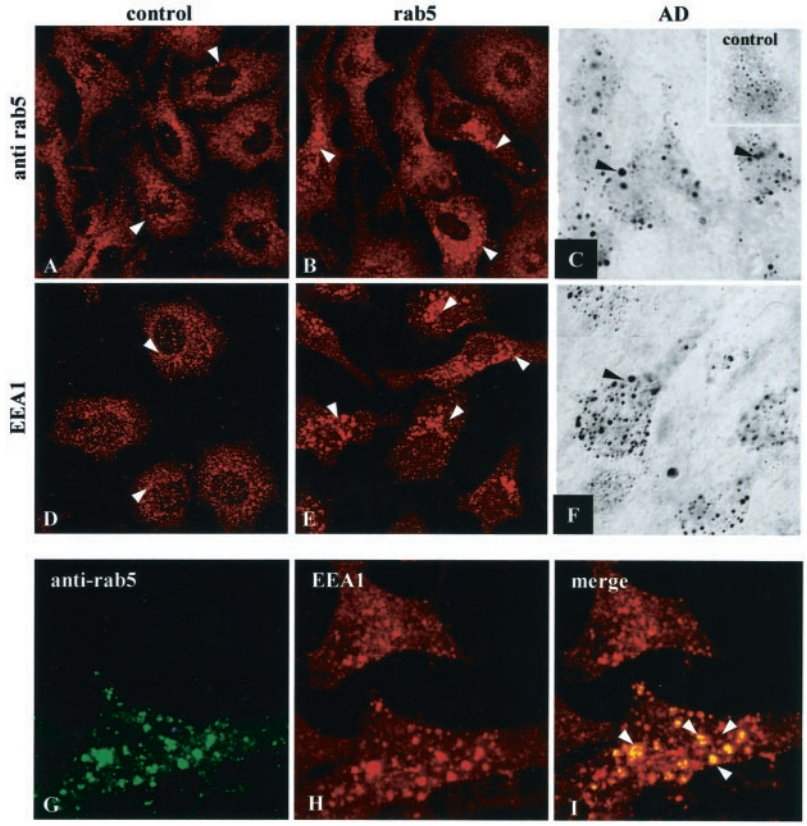

FIG. 2. **Rab5 overexpression mimics the endosomal enlargement seen in sporadic AD.** Immunofluorescence labeling of cells with antibodies to Rab5 (*A–C*; anti-Rab5 polyclonal antibody that recognizes both the mouse and human proteins) or to EEA1 (*D–F*) showed that control cells contain many small early endosomes of uniform size, distributed throughout the cell (*arrows*, *A* and *D*, respectively). The cells transfected with Rab5 (1.0 μg Rab5 cDNA/5 $\times$ 10^5 cells) contained atypically large Rab5-immunopositive (*B*) and EEA1-immunopositive early endosomes (*E*, *arrows*), which are morphologically similar to those seen in AD brain (*C* and *F*). Enlarged EEA1-immunoreactive early endosomes were found in cells overexpressing Rab5 by co-immunolabeling with the human-specific Rab5 mAb (*G*) and EEA1 (*H*).

system to an effect on the endocytic pathway. Earlier studies have shown that Rab5 overexpression increases the size of early endosomes (33). More detailed confocal analysis of cells immunolabeled with an anti-Rab5 antibody that recognizes both the mouse and human protein revealed small, uniform vesicular compartments in the control cells (Fig. 2*A*). In contrast to the control, Rab5 transfected cells contained numerous atypically large Rab5-positive vacuolar compartments (Fig. 2*B*), which were morphologically similar to those seen in neurons from AD brain (Fig. 2*C*). This morphologic alteration in early endosomes was confirmed using a second early endosomal marker, EEA1, both in the Rab5 transfected cells and in sections of human brain (Fig. 2, *D–F*). The pattern of endogenous EEA1 labeling in Rab5 overexpressing cells (Fig. 2*E*) closely resembles the pattern of enlarged early endosomal labeling seen in AD brain (Fig. 2*F*; see also Refs. 29 and 30). Moreover, we confirmed that the enlarged EEA1-immunoreactive early endosomes found in cells overexpressing Rab5 were co-immunolabeled with the human specific Rab5 mAb (Fig. 2*G*) and EEA1 (Fig. 2*H*).

We next examined the functional consequences of Rab5 overexpression by evaluating its effect on receptor-mediated and fluid phase endocytic uptake (Fig. 3). Incubation of living cells in the presence of fluorophore-tagged transferrin showed a qualitative increase in the number of transferrin-positive vesicles (Fig. 3*B*) in the Rab5 transfected cells (Fig. 3*C*) when compared with control cells (Fig. 3*A*), consistent with Rab5 overexpression stimulating receptor-mediated uptake (33). To look for similar changes in fluid phase uptake, we followed soluble HRP uptake (33). Ultrastructural examination of con-

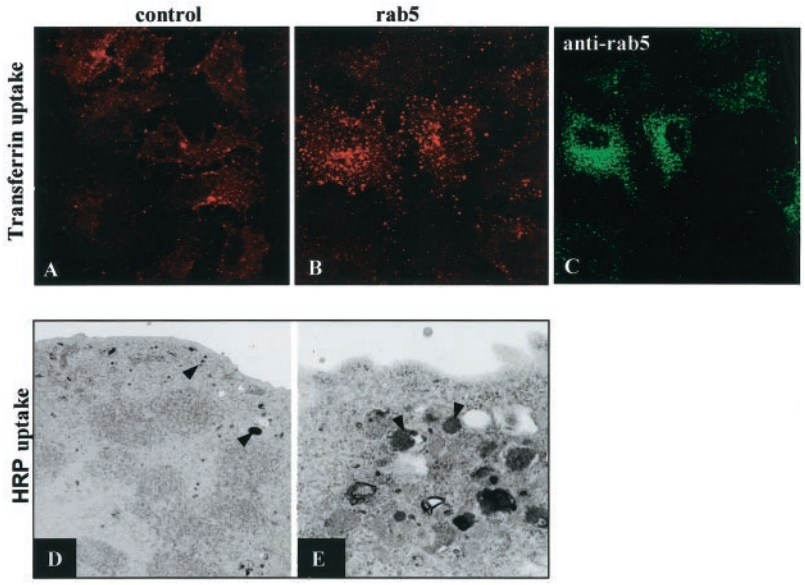

FIG. 3. **Rab5 overexpression stimulates receptor-mediated and fluid phase endocytosis.** Confocal fluorescent imaging of receptor-mediated endocytosis following a 3-min incubation of living cells with Cy3-labeled transferrin (*red*) showed a qualitative increase in the number and size of transferrin-positive vesicles (*B*) in human Rab5 overexpressing cells (*C*) when compared with the control cells (*A*). Ultrastructural inspection of fluid phase endocytosis following a 3-min incubation of cells with HRP showed numerous large HRP-positive vesicles close to the cell surface (*E*, *arrow*). Control cells contained much smaller electron dense vesicles (*D*, *arrow*).

trol and transfected cells incubated in HRP for up to 15 min showed that differences in endocytic uptake between control and Rab5 overexpressing cells were greatest at 3 min (data not shown). At 3 min, Rab5 overexpressing cells contained numerous large, electron-dense, HRP-positive endosomes (Fig. 3*E*, *arrows*) in close proximity to the plasmalemma, whereas control cells contained only smaller, HRP-positive vesicles (Fig. 3*D*).

Rab5 Overexpression Increases the Production of Aβ—Having determined that Rab5 overexpression mimics the morphological changes in early endosomes seen in neurons from AD and Down's syndrome brain, we next sought evidence that these endocytic alterations would impact APP metabolism. Initially, we determined the levels of human Aβ40 and Aβ42 in conditioned medium from both control and Rab5 transfected cells by sandwich ELISA in three independent experiments. When transfected with 0.5 μg of Rab5 cDNA/5 × 10^5 cells, cells produced over a 40-h incubation 1.8-fold more Aβ40 and 1.6-fold more Aβ42 when compared with control cells ($p < 0.05$ for both Aβ40 and Aβ42; Fig. 4*A*). With increasing amounts of Rab5 cDNA used for transfection, we recognized a trend toward greater Aβ40 and Aβ42 production, reaching 2.6 times more Aβ40 and 2.1 times more Aβ42 at 3.0 μg of Rab5 cDNA/5 × 10^5 cells. The increase in Aβ production associated with increasing amounts of Rab5 cDNA correlates well with the percentage of cells expressing human Rab5 at each cDNA amount (~45% at 0.5 μg, ~55% at 1.0 μg, and ~70% at 3.0 μg/5 × 10^5 cells). We confirmed that Aβ in the growth medium was increased in Rab5 transfected cells using a different antibody (4G8) and immunoprecipitation following a 6-h continuous metabolic labeling (Fig. 4*B*). When metabolically labeled Aβ was immunoprecipitated from cell lysates, a significant increase in intracellular Aβ was also reproducibly seen in the Rab5 overexpressing cells. These findings argue that Rab5 overexpression results in increased Aβ secretion and that this increase in extracellular Aβ is likely to be secondary to an increase in intracellular Aβ production.

Rab5 Overexpression Alters APP Processing—We next examined the turnover of APP holoprotein. Three independent pulse-chase immunoprecipitation experiments showed that APP holoprotein turnover was not significantly affected by Rab5 overexpression. Fig. 5*A* shows one such experiment, where the majority of APP holoprotein was degraded in both control and Rab5 expressing cells within 1 h. That Rab5 overexpression substantially increased Aβ generation without having a significant impact on APP holoprotein turnover is consistent with recent reports from our laboratory showing increased Aβ generation resulting from the manipulation of 46-kDa mannose 6-phosphate receptor expression or the calpain system without a change in APP turnover (31, 42).

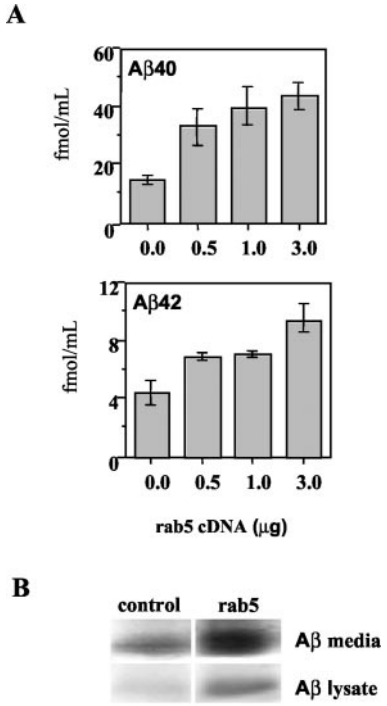

FIG. 4. **Rab5 overexpression increases the production of Aβ.** As quantitated by ELISA, Aβ40 and Aβ42 levels secreted into the growth medium over 40 h were significantly higher in cells transfected with increasing amounts of Rab5 cDNA as compared with control. The values represent the means ± S.D. of three independent experiments (*A*). This result was confirmed by 4G8 immunoprecipitation of labeled Aβ from medium and cell lysates following a continuous 6-h metabolic labeling of control or Rab5 transfected (1.0 μg of cDNA/5 × 10^5 cells) cells (*B*).

Next, we examined the relative amounts of soluble APP cleaved at either the α- or β-site (sAPPα and sAPPβ, respectively) in the growth medium of control and Rab5 overexpressing cells (1 μg of Rab5 cDNA/5 × 10^5 cells; Fig. 5*B*). Sequential

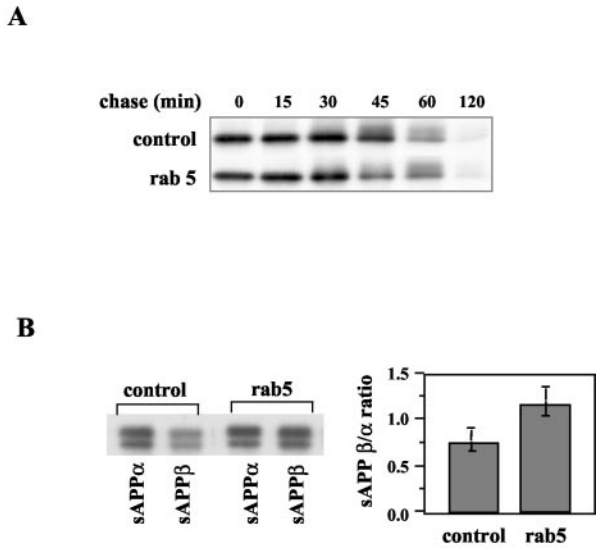

FIG. 5. **Rab5 overexpression does not affect APP metabolism but increases sAPPβ levels.** *A*, control and Rab5 transfected (1 μg of Rab5 cDNA/5 × 10^5 cells) cells were pulse-labeled for 15 min and chased as indicated prior to immunoprecipitation with C1/6.1 to show the turnover of APP holoprotein (42). There was no appreciable difference in APP turnover as a result of human Rab5 overexpression. *B*, sAPPα and sAPPβ were immunoprecipitated from conditioned medium following 1 h of chase. Quantification of three independent experiments revealed a 36% increase ($p < 0.05$) of the sAPPβ/sAPPα ratio in the medium of the Rab5 transfected cells when compared with control.

immunoprecipitation of the medium with 6E10 followed by P2-1 to detect sAPPα and sAPPβ was done initially as a pulse-chase experiment to determine the optimal time period for sAPP recovery (data not shown). Subsequent experiments were done using a 15-min pulse labeling with the chase medium collected after 1 h, which was found to reproducibly recover the sAPPα and sAPPβ generated from the labeled APP. A representative experiment is shown in Fig. 4*B*. Because L/APP cells express two forms of APP molecule, mouse and human APP695, we usually see sAPP running on the gel as a doublet. Quantitation of this experiment and two additional experiments demonstrated a reproducible increase in sAPPβ relative to sAPPα levels (mean increase, 36%; $p < 0.05$), in Rab5 overexpressing cells as compared with control.

β-Cleavage of APP is generally thought to be the rate-limiting step in Aβ production (2). Therefore, to determine levels of βCTFs in control and cells overexpressing Rab5, we designed a sensitive and quantitative sandwich ELISA taking advantage of the monoclonal antibody JRF/AβN/25 (Table I) and its specificity for β-cleaved APP metabolites (42). The sensitivity and linearity of this βCTF ELISA against a synthetic peptide is presented in Fig. 6*A* (*left panel*). In addition, we have develop a second ELISA that detects all transmembrane APP metabolites (APP/total CTF ELISA) by combining C1/6.1 capture with detection using a second carboxyl-terminal APP antibody, C2/7.1 (Table I). This ELISA showed linear detection of a GST-APP fusion protein containing the 99 carboxyl-terminal residues of APP (46) over a wide range of concentrations (Fig. 6*A*, *right panel*). Importantly, JRF/AβN/25 failed to react by ELISA with the GST-APP fusion protein because this construct does not have a free amino terminus at the Asp-1 residue of the APP fragment.

We have used these ELISAs to determine the levels of cell-associated βCTFs while simultaneously correcting for variations in cell density. In three independent experiments, Rab5 overexpression resulted in an increase in βCTF levels relative to APP holoprotein/total CTF levels (Fig. 6*B*). As was seen with Rab5 expression and Aβ secretion, increasing amounts of Rab5 cDNA used for transfection correlated with increasing relative levels of βCTFs, reaching a nearly 2-fold increase in βCTF levels as compared with control cells (3 μg of Rab5 cDNA/5 × 10^5 cells; $p < 0.01$).

When immunolabeled with JFR/AβN/25, both control and Rab5 transfected cells showed a similar pattern of intracellular, vesicular βCTF labeling (Fig. 6*C*). The level of βCTF immunosignal, however, was consistently greater in the Rab5 transfected cells, in agreement with our βCTF ELISA findings. To further characterize the distribution of βCTFs within cells, we did co-immunolabeling using markers of the TGN and early endosomes (Fig. 7). We found that although much of the βCTFs co-localized with a marker of the TGN (Fig. 7*A*), a significant βCTF immunolabeling was also found in early endosomal compartments identified with either EEA1 (Fig. 7*B*) or Rab5 (Fig. 7*C*). The partial coincidence of βCTFs and early endosomal markers is consistent with a key role for the early endosome in Aβ generation as well as a role for endocytic pathway up-regulation in increased Aβ production.

DISCUSSION

In our study we found that overexpression of Rab5 in L/APP cells increases internalization, both fluid phase and receptor-mediated, via the endocytic pathway and that this stimulation of endocytosis results in the enlargement of early endocytic compartments within the cell (32–34, 41). These enlarged early endosomes share many morphological features with the abnormal early endosomes in neurons of AD brain (Fig. 2) and Down's syndrome (29, 30). The significance of these endosomal alterations to AD pathogenesis is underscored by the disease specificity of this response (29), the importance of endosomes for APP processing (7–9, 47), and the influence of established genetic causal and risk factors such as APP[2] and ApoE4 (30) on the development of endosome alterations. Although dynamic analysis of neuronal endocytic activity cannot be done using human post-mortem tissue, significant circumstantial evidence argues that the morphological changes seen in the neuronal endocytic system are likely to reflect increased endocytosis. Many of the key positive regulators of endocytosis such as Rab5 and proteins that interact with Rab5 such as EEA1 (38–40, 48) and rabaptin 5 (35–37) are recruited to the enlarged endocytic vesicles of AD neurons (29, 30). We have also seen that Rab4 protein expression is increased in AD brain (29). Rab4 plays a reciprocal role to Rab5, stimulating the recycling of endocytic membrane and contents back to the cell surface (34, 49, 50). Additionally in AD, lysosomal hydrolase biosynthesis and delivery to early endosomes is increased, which is likely to have an impact itself on AD pathology (30, 31) and is a further reflection of an up-regulation of the whole endocytic machinery (29, 51). These neuronal endocytic changes appear years to decades before the onset of clinical disease (29) and therefore may play a early and ongoing role in the development of pathology.

To begin to test this hypothesis, we have mimicked some of these alterations *in vitro* and examined the impact on APP metabolism and Aβ production (Refs. 31 and 42 and this study). Here, we manipulated the endocytic system by Rab5 overexpression to increase endocytosis and fusion of homotypic early endosomes. Although not a complete model of the endocytic alterations in AD, Rab5 protein expression appears to be increased in affected neurons in AD (30, 52) and in a partial mouse model of Down's syndrome, ts65Dn, where neuronal endosomal enlargement was also observed.[2] Moreover, Rab5

[2] Cataldo, A. M., Petanceska, S., Peterhoff, C. M., Terio, N. B., Epstein, C. J., Villar, A., Carlson, E. J., Staufenbiel, M., and Nixon, R. A. (2003) *J. Neuroscience*, in press.

TABLE I
Key monoclonal antibodies directed against APP and/or specific APP metabolites used in this study

Antibody	Epitope specificity	APP metabolites detected
C1/6.1	Carboxyl-terminal 20 residues of APP	APP holoprotein and all CTFs
C2/7.1	Carboxyl-terminal	APP holoprotein and all CTFs
JFR/AβN/25	Residues 1–7 of Aβ; requires β-clevage	βCTF
6E10	Residues 1–17 of APP	APP holoprotein, sAPPα
P2–1	Amino-terminal lumenal domain of APP	sAPP

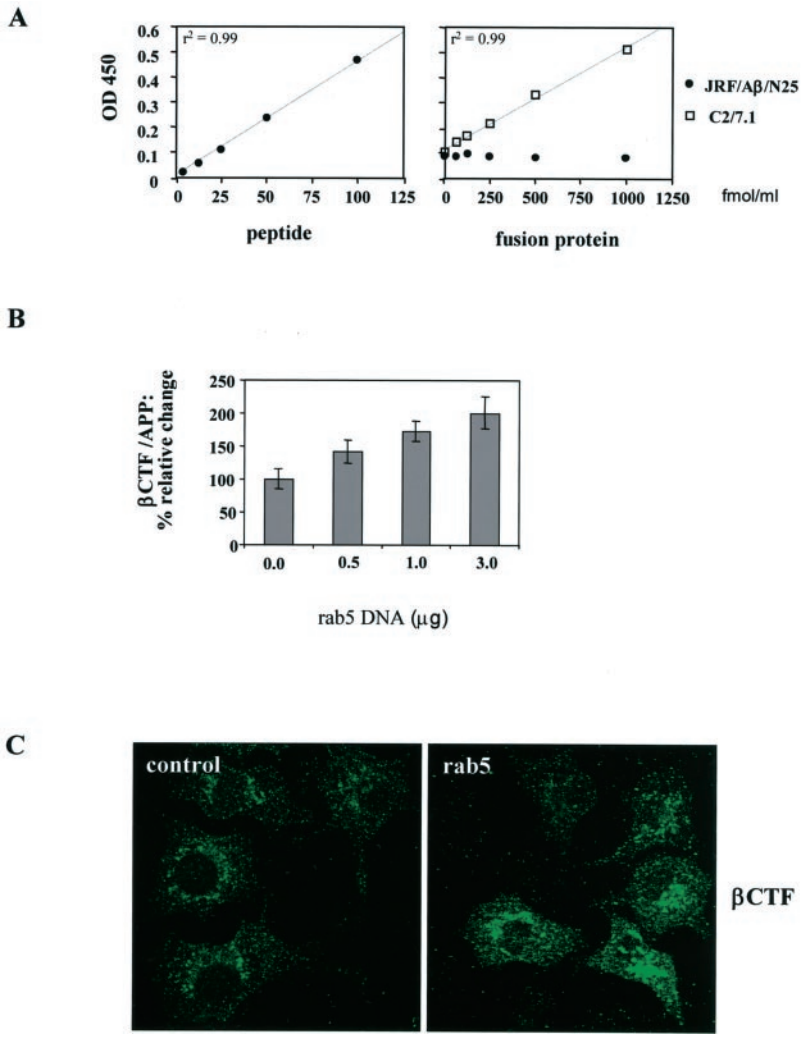

FIG. 6. **Rab5 overexpression increases levels of βCTF in the cell.** *A*, *left panel*, the sensitivity and linearity of the βCTF ELISA against a synthetic peptide standard is shown (see "Materials and Methods"). The linear detection of a GST-APP fusion protein containing the 99 carboxyl-terminal residues in the APP/total CTF ELISA (using C2/7.1 for detection) is also shown (*A*, *right panel*). Additionally, this graph demonstrates the βCTF specificity of JRF/AβN/25 as it fails to react with the GST-APP fusion protein, a construct that does not have a free amino terminus at the Asp-1 residue of the APP fragment (for details, see "Materials and Methods"). *B*, these ELISAs were used to determine the levels of βCTFs in cell lysates prepared from cells transfected with increasing amounts of Rab5 cDNA as indicated. The mean result of three independent experiments, expressed as the ratio of βCTF levels to APP/total CTF levels, showed an almost 2-fold increase following Rab5 overexpression as compared with control. *C*, this increase in βCTF levels was also revealed by immunocytochemistry using JFR/AβN/25 mAb where more βCTF immunolabeling was found in human Rab5 overexpressing cells as compared with control cells.

overexpression successfully reproduces many of the morphological changes seen in endosomes in AD neurons and thus allows one to examine the impact of at least this one important aspect of the endocytic alterations on proteins relevant to AD pathobiology. Understanding the consequences of endosome enlargement is a key first stage in clarifying the suspected pathogenic importance of altered endosomes in AD.

Accelerated Endocytosis and Aβ Production—α-Cleavage, which occurs within the Aβ peptide, prevents the production of Aβ from an APP molecule (2). Most experimental evidence suggests that α-cleavage occurs primarily at the cell surface and is the result of the activity of two metalloproteases (TACE and ADAM10) (53–58). Manipulations that increase the cell surface distribution of APP, such as estrogen treatment (59, 60) and APP phosphorylation by protein kinase C (61, 62), increase α-cleavage and reduce Aβ generation. Similarly, the removal (9) or replacement (7) of the APP cytoplasmic tail internalization motif (8) reduces Aβ generation, as does interfering with Rab5-mediated endocytosis more broadly (47). The fact that each of these manipulations reduces the endocytosis of APP and reduces Aβ production argues that endocytosis is critical for Aβ generation.

The positive activation of endocytosis by Rab5 overexpression, however, is likely to enhance APP internalization and thus reduce cell surface α-cleavage and the generation of sAPPα, as we have observed. Moreover, Rab5-stimulated endocytic activity resulted in a significant increase in sAPPβ and βCTFs levels, supporting a direct role for the early endosome in the amyloidogenic processing of APP via β-cleavage. Given that BACE, the protease responsible for β-cleavage, has been localized in part to the early endosome (3, 14, 15, 63), increased internalization of APP might increase the frequency of β-cleavage within this compartment. We have also shown that βCTFs partially co-localize with early endosomal markers both in control conditions as well as following manipulations that favor βCTF generation, such as Rab5 overexpression (Fig. 7) and calpain inhibition (42).

In addition to increasing βCTF levels, Rab5 overexpression promotes an equivalent rise in Aβ40 and Aβ42. This increase is similar in magnitude to the increase in βCTF levels, suggesting

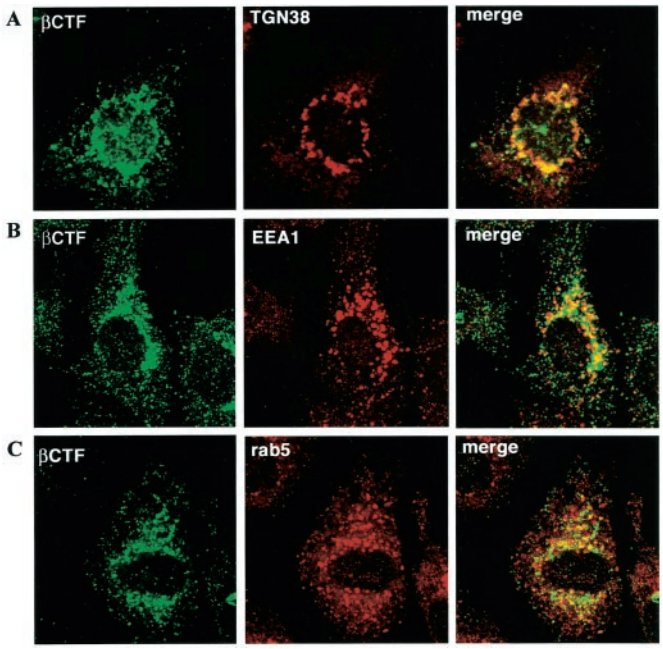

FIG. 7. **βCTFs partially co-localize with markers of early endosomes.** Confocal immunofluorescence microscopy showed that much of the βCTF (*green*) immunosignal was co-localized with TGN38 (*red*), a marker of the trans-Golgi network (*A*). However, significant βCTF immunolabeling was also found in early endosomal compartments (*red*) identified with either EEA1 (*B*) or anti-Rab5 antibody that recognizes both human and mouse protein (*C*).

that a direct precursor to product relationship may determine the increase in Aβ. Although additional studies will be required to establish whether γ-cleavage can also take place in early endosomes, our findings are most consistent with Rab5 overexpression leading to increased β-cleavage of APP in early endosomes, to increased βCTF levels, and ultimately to increased Aβ. In addition to extending previous studies demonstrating the importance of the endocytic pathway in APP uptake and processing (7–9, 47), our studies now provide a pathogenic link between the up-regulation of neuronal endocytosis in AD and increased Aβ generation.

Endocytic Pathway Up-regulation and AD Pathogenesis—In familial, early onset forms of AD, mutations in the APP or presenilin proteins have been shown to increase production of Aβ or the generation of particularly pathogenic forms of this peptide (reviewed in Ref. 2). Although it is generally thought that, in sporadic AD, some event or events leads to altered Aβ metabolism, such as increased generation, enhanced nucleation, and/or reduced turnover, the *in vivo* mechanisms underlying Aβ accumulation in sporadic AD are not well understood. Converging lines of evidence, however, now suggest that the endosomal system may play an important part in the complex etiology of sporadic AD and may have a direct role in the Aβ production that drives amyloid accumulation. First, the observations that endocytic pathway up-regulation and early endosome alterations are the earliest known neuronal changes to occur in AD and are highly disease-specific heightens suspicion that these abnormalities are pathogenically important (29, 30). Second, in preclinical AD and Down's syndrome, the appearance of enlarged neuronal early endosomes coincides with an increase in soluble Aβ levels and the detection of intraneuronal Aβ, which was found most frequently within enlarged endosomal compartments.[2] In this study, we show that Rab5 overexpression, which resulted in similar morphological changes in the endosome, increased Aβ production. Third, we have shown that Aβ is increased with overexpression of the 46-kDa mannose 6-phosphate receptor, which mimics an aspect of early stage AD endosomal pathology involving the partial mistrafficking of lysosomal hydrolases (31). Finally, individuals with presenilin mutations, who have very substantial β-amyloid burdens, do not show neuronal endosomal abnormalities, arguing that endosomal abnormalities in sporadic AD are unlikely to be a response to rising Aβ levels or β-amyloid deposition (29). Collectively, these findings strongly suggest that endocytic activation increases neuronal Aβ production in sporadic AD and may thus contribute to the accumulation of soluble or aggregated forms of Aβ peptide.

A complementary line of evidence is also developing that links the endocytic pathway to known genetic and other risk factors for AD. For example, the initial step in neuron metabolism of extracellular cholesterol is receptor-mediated uptake through endosomes. High levels of cholesterol itself can modify APP metabolism to favor Aβ production, both *in vitro* and *in vivo* (47, 64–66). ApoE, the major lipid carrier protein in the central nervous system, shuttles cholesterol produced by glia to neurons for endocytosis and clearly plays a role in AD pathogenesis given that inheritance of the E4 allele is the most important known risk factor for late-onset AD (67). Significantly, inheritance of the ApoE4 allele also accentuates neuronal endocytic abnormalities in sporadic AD (29). Although many mechanisms have been suggested to play a part in the risk associated with an ApoE4 allele (68, 69), these findings suggest a direct link to alterations in the endocytic pathway. An additional genetic link between AD pathology and AD-related endocytic pathway up-regulation is seen in Down's syndrome, which inevitably leads to AD in the fourth or fifth decade of life (70, 71). Beginning before birth, endosomal enlargement develops within increasing numbers of neurons in trisomy 21 individuals as they approach the age of AD onset (29). Thus, at least two known genetic influences on the rate-of-onset of AD significantly impact the neuronal endosomal system. Our findings suggest that the appearance of endosomal abnormalities in sporadic AD may increase Aβ production *in vivo*, contributing to a cascade of events that would lead to Aβ accumulation as β-amyloid plaque. Developing further *in vitro* and *in vivo* models of AD-related endocytic pathway up-regulation will be critical to formulating this link.

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