Memapsin 2 (\(\beta\)-secretase) is the protease that initiates cleavage of amyloid precursor protein (APP) leading to the production of amyloid-\(\beta\) (A\(\beta\)) peptide and the onset of Alzheimer’s disease. Both APP and memapsin 2 are Type I transmembrane proteins and are endocytosed into endosomes where APP is cleaved by memapsin 2. Separate endocytic signals are located in the cytosolic domains of these proteins. We demonstrate here that the addition of the ectodomain of memapsin 2 (M2ED) to cells transfected with native APP or APP Swedish mutant (APPsw) resulted in the internalization of M2ED into endosomes with increased A\(\beta\) production. These effects were reduced by treatment with glycosylphosphatidylinositol-specific phospholipase C. The non-transfected parental cells had little internalization of M2ED. The internalization of M2ED was dependent on the endocytosis signal in APP, because the expression of a mutant APP that lacks its endocytosis signal failed to support M2ED internalization. These results suggest that exogenously added M2ED interacts with the ectodomain of APP on the cell surface leading to the internalization of M2ED, supported by fluorescence resonance energy transfer experiments. The interactions between the two proteins is not due to the binding of substrate APPsw to the active site of memapsin 2, because neither a potent active site binding inhibitor of memapsin 2 nor an antibody directed to the \(\beta\)-secretase site of APPsw had an effect on the uptake of M2ED. In addition, full-length memapsin 2 and APP, immunoprecipitated together from cell lysates, suggested that the interaction of these two proteins is part of the native cellular processes.

Existing evidence supports the hypothesis that the initiation of Alzheimer’s disease (AD) is intimately related to an excess level of amyloid-\(\beta\) (A\(\beta\)) in the brain. The neurotoxicity of excess A\(\beta\) leads to the death of neurons, inflammation of the brain, dementia, and AD (1, 2). Because A\(\beta\) occupies such a central role in AD pathogenesis, the cellular mechanism of its genesis and the therapeutic means of its reduction are active areas of current research. A\(\beta\), a 40/42-residue peptide, is derived from the cleavage of amyloid precursor protein (APP), a Type I membrane protein (see Fig. 1), by two proteases known as \(\beta\)-secretase and \(\gamma\)-secretase. The activity of \(\gamma\)-secretase is associated with a membrane protein complex consisting of presenilin-1, nicastrin, and other proteins (3). \(\beta\)-Secretase was identified to be a membrane-anchored aspartic protease named memapsin 2 (4) (also BACE (5) and ASP-2 (6, 7)). Memapsin 2 is also a Type 1 transmembrane protein, consisting of a catalytic ectodomain (M2ED), a transmembrane domain, and a cytosolic domain (Fig. 1). The protease is synthesized in \(vivo\) with a 33-residue N-terminal pro-region, which is cleaved by furin en route to the cell surface (8–11). The crystal structure of M2ED (12, 13) is characteristic of aspartic proteases, consisting of two closely associated lobes with an extended substrate-binding cleft that accommodates eight substrate residues. The native APP is a poor substrate of memapsin 2 as judged by kinetic parameters (4, 14). The Swedish mutation at the P2-P1 positions, from Lys-Met to Asn-Leu, enhances the catalytic efficiency by about 60-fold, increases A\(\beta\) production, and manifests an early onset form of AD. The specificity of memapsin 2 for all eight substrate residues has been determined previously (15). Memapsin 2 is glycosylated by three N-linked oligosaccharides (16). The hinge that links the catalytic unit to the transmembrane region is only six residues long (12).

Memapsin 2 and APP are both located in “lipid rafts” on the cell surface where, at pH 7, memapsin 2 has only residual activity. Both proteins are endocytosed into the endosomes, the major site for the hydrolysis of APP by memapsin 2 (9, 11), leading to the production of A\(\beta\). The intracellular domain of memapsin 2 contains a signal motif that binds three GGA proteins (17, 18). This interaction apparently serves to package memapsin 2 into clathrin-coated vesicles for transportation in the endocytic or and recycling pathways (17, 18). Being located in the lipid raft at the plasma membrane (19, 20), memapsin 2 endocytosis is influenced by other components having direct or indirect contact with the protease. Such components include the glycosylphosphatidylinositol (GPI)-anchored proteins (19, 36), scramblase (21), heparan sulfate (22), and cholesterol (20). It has been shown recently that memapsin 2 and APP are in close contact on the cell surface and throughout the endocytic length memapsin 2; PBS, phosphate-buffered saline; CTF, C-terminal fragment; sAPP, soluble APP.
process (23). These observations suggest that the cell surface organization involving memapsin 2 may be important in the mechanism and regulation of memapsin 2 endocytosis, and contacts of other proteins with the catalytic domain of memapsin 2 may indeed play an important role in this process. We have therefore carried out a study of the endocytosis of exogenously added M2ED. Here we report that the recombinant M2ED is endocytosed, and this process requires the presence of APP at the cell surface. The internalized M2ED, localized mainly in endosomes, catalyzed the production of Aβ.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Two cell lines were used in the studies reported herein. Human embryonic kidney (HEK293) and HeLa cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 2% penicillin/streptomycin (10,000 IU/ml and 10,000 μg/ml, Cellgro), designated as complete DMEM. HeLa cells were used in all cell imaging experiments, whereas HEK293 cells were used whenever the overexpression of APP and memapsin 2 was needed for determination of APP degradation products. Human native APP (APPwt) and Swedish mutant APP (APPsw) were cloned in the mammalian expression vector pcDNA3.1 (Invitrogen) as APPwt/pcDNA and APPsw/pcDNA, respectively. The APP mutant lacking the endocytic motif GYENPTY (APP-s) was generated by site-directed mutagenesis (Stratagene) using APPsw/pcDNA as template. Full-length memapsin 2 (pro-M2) was cloned into pSecTag (Invitrogen) as M2/pSecTag. HER293 or HeLa cells were stably transfected with APPsw/pcDNA and/or M2/pSecTag designated as 293/APPsw or 293/M2/pcDNA, respectively. HeLa cells were stably transfected with APPsw/pcDNA designated as HeLa/APPsw. Antibiotic G418 in the concentration of 125 μg/ml was included in the media for maintenance of the stably transfected lines. For transient transfection, HER293 cells were seeded on 6-well plates, cultivated to 90–95% confluence, and 0.5 μg of APPwt/pcDNA, APPsw/pcDNA, APP-s/pcDNA, or empty vector (pcDNA3.1) were transfected for 24 h. Both stable and transient transfections were performed using LipofectAMINE 2000 (Invitrogen).

**Fluorescence Labeling of M2ED and Culture Incubation**—For fluorescent labeling of M2ED and pre-M2, fast protein liquid chromatography-purified recombinant M2ED and pro-M2ED (12) were separately dialeyzed with 0.1 M sodium bicarbonate buffer, pH 8.3, and labeled with either Alexa 488 fluorescent dye using an Alexa Fluor 488 protein labeling kit (Molecular Probes) or Cy3 using Cy3 Mono-reactive Dye Pack (Amersham Biosciences) following the manufacturer's recommended protocols. Labeled M2ED and pre-M2 retained its specific activity as compared with the native protease (results not shown) when assayed with a fluorogenic substrate (14), indicating that the modification did not significantly alter their three-dimensional structure. For cellular uptake of Alexa 488-labeled M2ED parental or APP-transfected HeLa cells were separately seeded on Lab-Tek™ 8-well chamber slides in complete DMEM or supplemented with 125 μg/ml G418 at 37 °C for 48 h. When about 70% confluent, the cells were washed once with pre-warmed DMEM, then 0.2 ml of a solution containing 9 μg of Alexa 488-labeled recombinant M2ED was added to each well and incubated for 30, 60, or 120 min at 37 °C. Cells were then washed with phosphate-buffered saline (PBS) 3 times, fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, and mounted withVectashield (Vector Laboratories Inc.) for observation with confocal microscopy. When the β-secretase inhibitor OMO0–3 was used, Alexa 488-labeled M2ED (1 μM) was preincubated with OMO0–3 (12.4 μM) for 30 min at 37 °C before adding to cells. The inhibition constant of OMO0–3 for M2ED at pH 7.0 was determined using the previously described method (15). The internalization of Alexa 488-labeled recombinant M2ED also was studied by flow cytometry as follows. APP-transfected 293 cells were seeded in T75 flasks (Nunc™, Denmark) and maintained in complete DMEM with 125 μg/ml G418 at 37 °C. At 90% confluence, the cells were trypsinized and centrifuged at 900 rpm for 10 min at 4 °C. The cell pellets were resuspended in 2 ml of Opti-MEM I medium (GIBCO™, Invitrogen) and equally divided into four parts onto 35-× 10-mm dishes (about 4 × 106 cells/dish). Each part was supplemented with 0 or 42 μg of Alexa 488-labeled recombinant M2ED or/and 0 or 2.5 units of glycosylphosphatidylinositol (GPI)-anchored protein (PI-PLC) and incubated at 37 °C or 4 °C, in the dark for 2 h each. Cells were washed with ice-cold 0.5% Tween 20/PBS and resuspended in 0.5 ml of PBS on ice for flow cytometric analysis (FACScalibur). For FRET experiment, HeLa/APPsw cells were incubated with 7 μg of M2ED-Cy3 in 0.2 ml of complete DMEM, and the rest of the procedure is the same as described above.

**Subcellular Localization Experiments**—APPsw-transfected HeLa cells were grown on 8-well chamber slides to 70% confluence and then were incubated with Alexa 488-labeled M2ED for 2 h as described above. Cells were rinsed with PBS three times, fixed with 4% paraformaldehyde in PBS at room temperature for 10 min, and permethylized for 6 min with PBST (PBS with 0.2% Tween 20). Cells were incubated with 5% host serum in PBS for 1 h at room temperature to block nonspecific protein binding sites and incubated for an additional 1 h with the respective primary antibodies against the following proteins: an early endosome marker EEA1 (in 1:2000 dilution, BD Biosciences), an endoplasmic reticulum marker, Bip/GRP78 (in 1:1500 dilution, BD Biosciences), a trans-Golgi network marker p230 (in 1:1500 dilution, BD Biosciences), and an anti-APP antibody, MAB1560 (in 1:700 dilution, Chemicon). The dilutions were carried out in 1% corresponding host serum in PBS. After extensive washes, Cy3-conjugated secondary sheep anti-mouse antibody (Sigma) was added. The cells were incubated for an additional 1 h, followed by extensive washes, and mounted with Vectashield mounting medium. Cellular images were collected with an LSM 510 META laser scanning confocal imaging system (Carl Zeiss) using multiple photomultiplier tubes regulated by LSM510 software. Alexa 488-labeled M2ED was excited at 488 nm, and emission was recorded at 505–530 nm. Cy3 was excited at 543 nm, and emission was
recorded at 560 nm. The captured images for the red and green channels were merged, with the appearance of yellow being indicative of colocalization.

**Internalization of M2ED by Cells Treated with Glycosylphosphatidylinositol-specific Phospholipase C—HeLa cells transfected with APPsw were grown in 8-well chamber slides to about 70% confluency then incubated with 0 or 2.5 units/ml of PI-PLC (Molecular Probes) in Opti-MEM I Reduced Serum Medium (Invitrogen) at 37 °C for 2 h.** The incubation of Alexa 488-labeled M2ED, and the observation of cell-associated fluorescence were carried out as described above.

**Cellular Production of Soluble APP, C-terminal Fragment, and β-HEK293 cells stably transfected with APPsw were methionine deprived for 1 h in a methionine-free DMEM (Cellgro) with 5% dialyzed fetal bovine serum and 25% DMEM. Then cells were incubated in 10% FCS containing 10 μM sulfonyl fluoride, 10 μM leupeptin, 2.5 μM EDTA, 1 μM pepstatin, and 0.23 unit/ml aprotinin) for 20 min on ice, followed by passing through a 21-gauge needle 10 times. The cells were centrifuged at 15,000 × g at 4 °C for 20 min. Protein concentrations of the lysates were determined by the Bio-Rad protein assay (Bio-Rad). Supernatant and cell lysates were immunoprecipitated, respectively, with anti-APP antibody (Rockland), anti-APP antibody (Affinity Bioreagents) against N-terminal 17 amino acids, and MAB1561 (Chemicon) against residues 1–24 of Aβ for detection of C-terminal fragment (CTF), soluble APP (sAPP), and Aβ. Antibody complex captured with 20 μl of protein-G-agarose beads (Sigma) were resolved by electrophoresis on 12–20% gradient Tris-Tricine polyacrylamide gels (Invitrogen). Gels were soaked in a fixation solution (20% methanol and 10% acetic acid) for 30 min followed by amplification solution Amplify NAMP100V (Amershams Biosciences) for 1 h then dried and exposed to BioMax film (Kodak).

**FRET Experiments—Fluorescence resonance energy transfer (FRET) experiments were performed using the acceptor photobleaching method (24) in which FRET induced an increase in fluorescence of the donor, anti-APP antibody conjugated to Alexa 488 (anti-APP-Alexa 488), as a consequence of the photobleaching of the acceptor, M2ED conjugated to Cy3 (M2ED-Cy3), was measured. APPsw-transfected HeLa cells grown in chamber slides were incubated with Cy3-labeled M2ED, fixed with paraformaldehyde, and permeabilized as described above. For APP detection, monoclonal antibody against APP, MAB1561, was labeled using the Alexa 488 Zenon® labeling kit (Molecular Probes), which specifically labeled the Fab fragment of the antibody. The extent of labeling was controlled, so the donor fluorescence intensity was considerably lower than that of the acceptor. The use of fluorescein-labeled M2ED as the donor was essential because whole IgG secondary antibodies failed to result in a FRET signal. Cells were incubated with 1 μg of Alexa 488-labeled anti-APP antibody in 5% normal goat serum/PBS at room temperature for 2 h. After washing five times with PBS, the cells were mounted in 50% glycerol in PBS. FRET imaging was performed using a Nikon C1 confocal scanning head mounted on a Nikon TE 2000 inverted microscope equipped with argon and He-Na green light lasers (emission lines: 488 nm and 568 nm, respectively). For photobleaching the He-Na laser was used at its maximum power. Consecutive images were acquired of donor and acceptor before bleaching using low laser energy. Thereafter, the M2ED-Cy3-stained cells were bleached in a region of interest by repeated scanning with the full power 568 nm laser, and the bleaching interval was 6–8 min, which reached 40–60% of the prebleach fluorescence. Post-bleached images were acquired by reverting back to the original settings. All measurements were obtained using a 60 × 1.4 numerical aperture objective, and samples labeled with the donor and acceptor alone were used to check the cross-over between the fluorophores. The gain of the photomultiplier tubes was adjusted so as to accommodate the donor signal without photobleaching wafers being observed. In addition, no FRET signal was detected when the Cy3-labeled Fab fragments were replaced with labeled whole IgG, suggesting that two IgG layers (the primary and secondary antibodies) increase the distance between fluorophores, and therefore FRET no longer occurs. This observation provides additional proof that the detected FRET signal accurately reflects the proximity of the two fluorophores.

**Immunoprecipitation of APP and Full-length Memapsin 2 from Cell Lysates—Parental 293 and 293/M2APPsw cells were separately grown to 90% confluence in T75 flasks. The cells were treated with 0 or 2.0 units/ml of PI-PLC in Opti-MEM 1 medium at 37 °C for 2 h, then washed with PBS and lysed in radioimmunoprecipitation assay buffer with protease inhibitors and 1% saponin (Calbiochem) for 20 min on ice. Total cell lysates were collected by centrifuging at maximum rpm at 4 °C for 20 min, divided into two parts, and immunoprecipitated with anti-APP antibody MAB1561 and anti-memapsin 2 antibody 195102 (Calbiochem) against residues 487–501 of human memapsin 2. The antibody complexes washed on protein-G beads were eluted in sample loading buffer (50% SDS-PAGE) (16%) Tris–Tricine and run on Western blotting with antibody 195102 or antibody MAB1561, respectively, following transfer to polyvinylidene difluoride membranes. Control blots of washed protein-G beads without added antibodies for immunoprecipitation produced very low nonspecific bands (results not shown), thus the specific immunoprecipitated bands are readily identifiable in this system.

**RESULTS**

**Endocytosis of Exogenously Added M2ED into HeLa Cells Is Dependent on APPsw Expression—Recombinant M2ED, co-valently conjugated with Alexa 488 fluorescent dye, was incubated with HeLa cells with or without transfection of APPsw. After 2 h, the cells were fixed and observed with confocal microscopy for the green fluorescence of the dye-conjugated M2ED. In native HeLa cells, little fluorescence was found to be associated with the cells, with only a few positive sites present on the cell surface (Fig. 2A). Cells transfected with a blank vector did not increase the cell-associated fluorescence (results not shown). For HeLa cells transfected with APPsw, intense punctate fluorescent areas were observed inside cells (Fig. 2B) and the intracellular fluorescence increased with the duration of incubation (Fig. 2C). The expression of native APP also stimulated the internalization of M2ED (see below). Similar results were also obtained when HEK293 cells were used (results not shown). To confirm the above observations, HEK293 cells were chosen (see “Discussion”) for experiments using flow cytometric analysis. HEK293 cells expressing APPsw manifested significantly higher cell-associated fluorescence after incubation at 37 °C with Alexa 488-labeled M2ED than did the native cells (Fig. 3). Incubation of 293/APPsw cells and M2ED at 4 °C had only marginal increase of cellular fluorescence (Fig. 3), because endocytosis is impeded at this low temperature. These findings suggest that exogenously added M2ED was internalized into the cells, and such process was facilitated by the presence of APPsw.

**Endocytosis of Exogenously Added M2ED Does Not Require an Available Active Site of Memapsin 2—We examined the possibility that APPsw facilitated endocytosis of the added M2ED by the formation of an enzyme-substrate complex. Such a complex would require the availability of memapsin 2 substrate binding site and the APPsw region near the β-secretase cutting site. Therefore, we studied the effect on M2ED internalization into HeLa cells by either a memapsin 2 inhibitor or an antibody directed at the β-secretase site of APPsw. Inhibitor OM00–3 is very potent (Ki = 0.3 μM at pH 4.5 (14)) and occupies all eight subsites in the memapsin 2 active-site cleft (12). At pH 7.0, the pH of the culture medium, OM00–3 has a Ki of 1.1 μM. The inhibitor did not significantly alter the uptake of Alexa 488-labeled M2ED by HeLa/APPsw cells (Fig. 2D), at about 12× molar excess to M2ED (calculated to abolish 92% of the proteolytic activity). Monoclonal antibody 1560, which recognizes residues 1–17 of Aβ, including subsites P4 to P1 of the β-secretase site of APPsw, was then tested for blocking of the binding of M2ED to this site of APPsw. The inclusion of this antibody did not change the uptake of exogenously added M2ED (Fig. 2E). These observations suggest that the APPsw-
mediated uptake of M2<sub>ED</sub> does not require the formation of an enzyme-substrate complex involving the active site of memapsin 2.

**Internalized M2<sub>ED</sub> Colocalized Mainly with Endosomes and APP.**—To determine the subcellular localization of internalized M2<sub>ED</sub>, APP<sub>sw</sub>-expressing HeLa cells were incubated for 2 h with Alexa 488-conjugated M2<sub>ED</sub> then permeabilized and stained with antibodies directed for resident proteins of various subcellular compartments. Confocal microscopy observed little colocalization between M2<sub>ED</sub> and endoplasmic reticulum marker protein BiP (Fig. 4A). Likewise, limited colocalization was seen for Golgi marker p230 (Fig. 4B). Strong colocalization involving much of the cytosol area was observed for early endosome marker EEA1 (Fig. 4C). With a shorter incubation (1 h) but otherwise the same experimental conditions, M2<sub>ED</sub>-associated fluorescence was still seen colocalized with endosome marker EEA1 but not with Golgi marker (results not shown).

Using the same experimental procedure as above, we also observed that the internalized M2<sub>ED</sub> colocalized with APP in a distribution pattern similar to that of the endosomes (Fig. 4D). To further substantiate this point, we employed a method based on fluorescence resonance energy transfer (FRET) to determine the proximity of protein-protein interactions (25, 26). HeLa/APP<sub>sw</sub> cells were incubated with Cy3-labeled M2<sub>ED</sub> for 2 h. After fixation and permeabilization, cells were immunostained with anti-APP antibody conjugated with Alexa 488. Photobleaching of the Cy3 label of M2<sub>ED</sub> led to a marked increase in the APP fluorescence signal specifically within the photobleached area but without significant changes in the non-bleached regions (Fig. 5). These observations indicated the transfer of fluorescence energy from internalized M2<sub>ED</sub> to APP and the two fluorescence-labeled proteins are in close proximity (within 10 nm). The FRET observed also met two established criteria for specificity. First, the FRET signal was totally absent in cells labeled with donor fluorophore, proving that the detected signal is not due to an unspecific dequenching, dependent on the autofluorescence of the tissue. Second, when the acceptor (Cy3) was photobleached in the region of interest, because less energy was absorbed by the acceptor in the bleached area, whereas non-bleached areas remained unchanged. These observations indicated the specificity of the FRET assay for detecting APP-M2<sub>ED</sub> interactions on the cell surface and within intracellular compartments. The amount of energy transfer, calculated as a percent increase of the average fluorescence intensity in the post-bleached region of interest, increased by 29.3 ± 2.3% (n = 10; p < 0.00001). Together, the above results demonstrate that internalized M2<sub>ED</sub> is mainly present in the endosomes and it colocalized with APP<sub>sw</sub> with a close molecular association between the two.

**Internalization of M2<sub>ED</sub> Is Influenced by Cell Surface GPI-anchored Proteins—**It has been shown that the removal of...
GPI-anchored cell surface proteins reduced the endocytosis of cellular expressed native memapsin 2 (19). We therefore sought to determine if the same effect is also seen for exogenously added M2ED. After the HeLa cells expressing APPsw were treated with PI-PLC, the internalization of Alexa 488-conjugated M2ED was substantially reduced, as seen in confocal microscopy, when compared with the untreated control (Fig. 6A). Quantification of cell-associated fluorescence indicated that the uptake of PI-PLC-treated cells was only about 10% of that for the untreated cells (Fig. 6B). These results were substantiated by flow cytometric analysis. PI-PLC treatment (2.5 units/ml) reduced the uptake of exogenously added M2ED compared with the control (Fig. 3A). These observations suggest that cell surface GPI-anchored proteins contribute to the efficiency of internalization of the added M2ED.

**M2ED Internalization Resulted in the Production of Aβ**—Because exogenously added M2ED was internalized to endosomal compartments with APPsw, we then investigated if the internalized M2ED could support the cleavage of APPsw to Aβ. M2ED was added to APPsw-transfected HEK293 cells. After metabolic labeling with [35S]methionine, Aβ and soluble APP (sAPP) secreted in the media and C-terminal fragment (CTF) in the cell lysate were separately immunoprecipitated and detected by 10–20% gradient SDS-PAGE and autoradiography. The addition of M2ED to the cells clearly produced significantly more Aβ, sAPP, and CTF (Fig. 6C, central lane) than the control (Fig. 6A, left lane). These observations indicate that internalized M2ED is active and hydrolyzes APPsw to produce Aβ. We also found that the treatment of phospholipase C, which diminished memapsin 2 endocytosis (Fig. 6, A and B) also diminished the production of Aβ, sAPP, and CFT in APPsw-expressing HEK293 cells with exogenously added M2ED (Fig. 6C, right lane). This observation also confirmed that internalized M2ED involved in the production of Aβ.

**Internalization of M2ED Is Dependent on the Endocytosis Motif GYENPTY in the Cytosolic Domain of APP**—The endocytosis of Type I membrane proteins is in general mediated by their cytosolic domains (for review, see Ref. 27), and a potential endocytosis signal for memapsin 2 is located in its cytosolic domain (11, 28). Without a cytosolic domain, M2ED should be devoid of an endocytosis signal. The dependence of the cellular uptake of M2ED on APPsw suggests that this internalization process may be mediated by the known endocytosis signal of APP, a motif with the sequence GYENPTY in the cytosolic domain (29, 30). We studied the effect of deletion of this motif in APP on the uptake of M2ED by cells. HeLa cells were transfected separately with vectors for the expression of wild type APP, APPsw, and APP deletion mutant (APP-s), and the internalization of Alexa 488-conjugated M2ED was observed with confocal microscopy. Images in Fig. 7 show that all vectors expressed at about the similar level as indicated by the staining of vector-derived myc epitope encoded at the C terminus of each APP construct, except the blank vector control (upper row). The deletion APP mutant (APP-s, lower right) had a dramatically lower uptake of M2ED but more cell surface distribution compared with the cells expressing either wild type APP or Swedish mutant APP (two central plates, lower row). The residual fluorescence level in cells expressing the blank vector (lower left) was considered only background. These observations suggest that the internalization of M2ED requires the endocytosis signal in the cytosolic domain of APP.

**Interaction of M2ED with APPsw on Cell Surface**—The results described above suggest that direct or indirect interaction of APP and M2ED on the cell surface is an early event leading to M2ED internalization. This hypothesis is consistent with the colocalization of the two proteins in cells (Fig. 4). Further substantiation of this aspect, came from FRET studies. HeLa/APPsw cells were incubated for a short time at 37 °C and a specific focal plane of the image was chosen to demonstrate the labeled M2ED on the cell surface. Destruction of the acceptor (Cy3) fluorescence by photobleaching leads to a corresponding increase in the donor (Alexa 488-labeled anti-APP antibody) emission intensity over the cell surface within the beached region of interest. This suggests that APP and M2ED are closely located within the spatial scale of FRET effect (<10 nm) (Fig. 8).

**Full-length Memapsin 2 Is in a Complex with APP in Cells**—We asked if the interaction between M2ED and APP described above also occurs with full-length memapsin 2. Lysates of HEK293 cells from either the parental control cells or cells cotransfected with APPsw and full-length memapsin 2 were separately prepared in the presence of 1% saponin to break up the lipid rafts. Lysates were immunoprecipitated with an antibody against APP and subjected to Western blots for memapsin 2 or vice versa. An APP band was clearly shown in immunoprecipitation of memapsin 2 from double transfected cells (Fig. 9A, left panel). The light APP band from the parental cells was likely derived from the endogenously expressed APP in HEK293 cells. Likewise, in the APP immunoprecipitation of lysates from transfected cells, a memapsin 2 band was clearly present (Fig. 9B, left panel). A weak memapsin 2 band representing the coimmunoprecipitation of endogenously expressed memapsin 2 and APP is also in the parental cell lysate. We also examined if the treatment of PI-PLC affected coimmunoprecipitation of APP and memapsin 2. When transfected cells were treated with PI-PLC, the coimmunoprecipitated APP (Fig. 9A, right panel) and memapsin 2 (Fig. 9B, right panel) were clearly
reduced. These results suggest that full-length memapsin 2 and APP directly interact in the cells, and this interaction is not a consequence of overexpression of transfected APP. The treatment of cells with PI-PLC that reduced the endocytosis of M2ED also reduced the cell surface interaction of APP and memapsin 2. The fact that APP appeared in SDS-gel as a single band suggests that the interaction with memapsin 2 involves the APP with the fully matured oligosaccharide, possibly on the cell surface and in the endocytic pathway.

**DISCUSSION**

The observations described above show that exogenously added M2ED is internalized to endosomes by cells, resulting in the production of Aβ. This internalization process is facilitated by the presence of APP on the cell surface, because little internalization of M2ED takes place unless the cells are transfected to express APP or APPsw (Figs. 1 and 6). Data also suggest that APP directly mediates the endocytosis of M2ED. Not only is M2ED not internalized when the endocytosis signal of APP is absent (Fig. 7), but also the positive FRET (Fig. 8) suggests that interaction with memapsin 2 involves the APP with the fully matured oligosaccharide, possibly on the cell surface and in the endocytic pathway.

**Fig. 5.** FRET analysis confirms the colocalization of intracellular M2ED and APPsw. M2ED was labeled with Cy3 (Cy3-M2ED, designated “A” for acceptor), and the Fab portion of the anti-APP antibody MAB1560 was labeled with Alexa 488 (designated “D” for donor). HeLa/APPsw cells were incubated with 7 μg of Cy3-M2ED in 0.2 ml of complete DMEM at 37 °C for 2 h. After fixation and permeabilization, cells were immunostained with Alexa 488-anti-APP. The fluorescence signal of APP was found in both cytosol and cell membrane and colocalized with the memapsin 2 signal in both areas. A and C are pre- and post-bleached images of the donor (Alexa 488-APP). B and D are corresponding acceptor (Cy3-M2ED) images of the same cell. The boxes indicate the bleached region of interest used for intensity measurements. E represents a pseudocolor encoding of the image obtained by pixel-by-pixel subtraction of donor prebleach (A) from the donor postbleach (C) images. F indicates the intensity of the acceptor and the donor. Photobleaching of the Cy3 label of memapsin 2 in either cytosol or cell membrane led to a marked increase in the APP fluorescent signal within the photobleached area, demonstrating FRET. Bar, 20 μm. Data are representative of three independent experiments.
length memapsin 2 and APP are immunoprecipitated together (Fig. 9), an indication that some of these two proteins are in association in the cells. A unified scheme extended from the current results is that the ectodomain of APP serves as a receptor for memapsin 2, or M2ED as a substitute, to internalize both proteins mediated by the endocytosis signal of APP. In the acidic endosome, some of the complex may dissociate and cleavage at the \( \gamma \)/H9252 site may then occur. An interesting consequence of such a scheme is that it provides various possible points at which the internalization of memapsin 2 and the production of A\( \beta \)/H9252 may be regulated. Superficially, the dependence on APP for memapsin 2 internalization appears to contradict with the fact that the cytosolic domain of memapsin 2 contains a potential endocytic signal. The endocytosis of memapsin 2 is thought to require its cytosolic domain and a dileucine motif contained therein (11, 26). It was demonstrated recently that the dileucine motif is part of the acid cluster-dileucine (ACDL) sequence DDISLL that binds to the VHS domain of GGA (Golgi-localized \( \gamma \)-ear-containing ADP-ribosylation factor binding) proteins (17, 18). Because GGA proteins also bind clathrin, this interaction appears to mediate the packaging of memapsin 2 to the coated pit for...
vesicular transport. The interaction of the ACDL motifs of mannose 6-phosphate receptors (M6PR) with GGA proteins is known to mediate the transport of lysosomal enzymes from the trans-Golgi to endosomes (32–34). The participation of ACDL in the endocytosis process at the cell surface has, however, not been demonstrated. In fact, the interaction of GGA proteins with the native memapsin 2 ACDL motif is more than 18-fold weaker (Kd ranging from 184 to 303 mM for three GGA proteins) (18) than the interaction between the ACDL motif of M6PR with GGA proteins (Kd of about 10 mM) (18, 35). On the other hand, the phosphorylated ACDL motif of memapsin 2, known to function in the recycling of memapsin 2 from endosomes back to cell surface (31), exhibits a Kd toward GGA3 of 22 nM (18), approaching the level of interaction between M6PR and GGA proteins. Therefore, the weak interaction of memapsin 2 ACDL with GGA proteins may be below the operational threshold, and the endocytosis of native memapsin 2 may then be dependent on the interaction of its ectodomain with APP. The current results could not exclude the possibility that the ACDL motif of memapsin 2 and the GYENPTY motif of APP are both used in endocytosis of memapsin 2 under different settings. Clearly, more experimental data will be required for sorting out these and other possible explanations.

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