Memapsin 2 (BACE, \(\beta\)-secretase) is a membrane-associated aspartic protease that initiates the hydrolysis of \(\beta\)-amyloid precursor protein (APP) leading to the production of amyloid-\(\beta\) (A\(\beta\)) and the progression of Alzheimer disease. Both memapsin 2 and APP are transported from the cell surface to endosomes where APP is cleaved by memapsin 2. We described previously that the cytosolic domain of memapsin 2 contains an acid cluster-dileucine motif (ACDL) that binds the VHS (Vps-27, Hrs, and STAM) domain of Golgi-localized \(\gamma\)-ear-containing ARF-binding (GGA) proteins (He, X., Zhu, G., Koelsch, G., Rodgers, K. K., Zhang, X. C., and Tang, J. (2003) Biochemistry 42, 12174--12180). Here we report that GGA proteins colocalize in the trans-Golgi network and endosomes with memapsin 2 and a memapsin 2 chimera containing a cytosolic domain of a mannose-6-phosphate receptor. Depleting cellular GGA proteins with RNA interference production. Another protease, \(\beta\)-secretase, a 40- or 42-residue peptide called amyloid-\(\beta\) (A\(\beta\)) is produced. An excessive level of A\(\beta\) in the brain facilitates a series of adverse events, including the accumulation of A\(\beta\) plaques, brain inflammation, and the death of neurons, and leads to the progression of Alzheimer disease (5). In view of its pivotal position in the pathogenesis of Alzheimer disease, memapsin 2 is viewed as a promising target for the development of inhibitor drugs to treat Alzheimer disease. In this connection, the understanding of molecular and cellular activity of memapsin 2 presently is of great scientific interest.

Memapsin 2 is a type I transmembrane protein with a single transmembrane segment linking a luminal catalytic ectodomain, which is homologous to other mammalian aspartic proteases (6), to a C-terminal cytosolic tail of 21 residues. Newly synthesized pro-memapsin 2 is processed to mature protease by furin during transit through the secretory pathway to the cell surface (7--10). Memapsin 2 and its substrate APP are both internalized from the cell surface to endosomes where APP is cleaved by memapsin 2 in an acidic medium, leading to the production of A\(\beta\). The memapsin 2 protease then is recycled to the cell surface (10, 11). Thus, the cellular trafficking of memapsin 2 is intimately related to the mechanism and regulation of A\(\beta\) production.

As in the case of many other membrane proteins, the cellular transport of memapsin 2 is mediated by its cytosolic domain (10, 12). Recently, the region of memapsin 2 cytosolic domain that mediates cellular trafficking has been traced to a motif with the sequence DISLL. This motif, which belongs to the acidic cluster-dileucine (ACDL) sorting signal (DXXLL, where \(X\) denotes a nonconserved residue) (for review, see Ref. 13), binds the VHS (Vps-27, Hrs, and STAM) domains of Golgi-localized \(\gamma\)-ear-containing ARF-binding (GGA) proteins (14, 15). GGA proteins are known to bind the ACDL-sorting signal in the cytosolic tails of mannose-6-phosphate receptors (MPRs) (16, 17) and other lysosomal membrane proteins (18) as the initial sorting recognition in the transport from trans-Golgi to endosomes. By analogy, the GGA/memapsin 2 interaction is thought to mediate the cellular transport of memapsin 2 (14, 15), although direct cellular evidence and the specific route of involvement have yet to be demonstrated. However, several pieces of evidence argue against the involvement of GGA proteins in the endocytic pathway of memapsin 2. First, GGA proteins are associated with trans-Golgi and endosomes but scarcely at the cellular plasma membrane (19, 20). Second, membrane 2 ectodomain devoid of GGA binding motif is internalized by cells through the interaction with APP (21), suggesting that the ACDL motif of memapsin 2 is not essential for its endocytosis. Third, the native ACDL motif of memapsin 2 interacts approximately 20 times weaker than the corresponding interaction with mannose-6-phosphate receptors. The phosphorylation of Ser\(^{498}\) in the ACDL motif of memapsin 2 enhances GGA binding to the intensity similar to that for MPR proteins (15). Phosphorylation of the memapsin 2 ACDL motif is essential for its recycling pathway from early endosomes to the cell surface (11). These observations open the possibility that the interaction of GGA proteins with the phosphorylated memapsin 2 may mediate the recycling of the protease from endosomes to trans-Golgi on the way back to the cell surface.
back to the cell surface. To further understand the involvement of GGA proteins in intracellular transport of memapsin 2, we studied the cellular trafficking of memapsin 2 and its chimera with MPR cytosolic domain. We report in this paper that GGA proteins are involved in the cellular transport of memapsin 2 from endosomes to the cell surface in the recycle pathway.

MATERIALS AND METHODS

cDNA Vectors—The constructs of expression vectors of human wild-type memapsin 2 and Swedish mutant of APP cDNAs were described previously (1). PCR-based mutagenesis was used for the constructs of memapsin2 mutants. In M2-MPR, memapsin 2 cytosolic domain from residue 481 to the C terminus was replaced with the cytosolic domain of CD-MPR (Fig. 1A) with the sequence QRLVVGAKGMEQFPHLAFWQ-
proteins, hemagglutinin-GGA1, GGA2-V5, and GGA3-V5, were described previously (15). The cDNA constructs encoding these mutants in memapsin 2 were inserted into the mammalian expression vector pSecTag2 (Invitrogen). Expression vectors for the GGA proteins in intracellular transport of memapsin 2, we cytosomal domain have been replaced by alanines thirds line, see C-terminal sequence with boldface letters A), schematic diagram of the domain organization of human GGA proteins. C–E, morphology of the HeLa cells separately expressing three vectors shown in A. The cells were fixed and permeabilized prior to immunocytochemical staining. The first antibody is the affinity-purified rabbit anti-promemapsin 2, and the secondary antibody is the Cy3-conjugated Sheep anti-rabbit antibody. Scale bar represents 20 μm.

antisera or mAbs to the rabbit polyclonal antibodies (Covance, Denver, PA) and goat polyclonal antibodies against recombinant pro-memapsin2 were affinity-purified using Affi-Gel (Bio-Rad) immobilized recombinant memapsin 2 (1). Monoclonal antibodies mAb1560 (specific to Aβ(1–17)) and rabbit polyclonal antibodies AB5352 (specific to APP C- terminal) were obtained from Chemicon (Temecula, CA). Mouse monoclonal antibodies for early endosome antigen 1 (EEA1), TGN38, Bip/GRP78, Lamp-1, GGA2, and GGA3 were purchased from BD Biosciences. Mouse monoclonal antibodies for the Golgi marker Giantin were purchased from Calbiochem. Monoclonal V5 antibody was purchased from Invitrogen. Monoclonal anti-hemagglutinin antibodies and Cy3-conjugated secondary donkey anti-mouse antibody were purchased from Sigma. Alexa 488-conjugated secondary donkey anti-mouse antibody was purchased from Molecular Probes. Rabbit polyclonal anti-β-actin and goat polyclonal anti-VPS26 were purchased from Novus Biologicals. Rabbit polyclonal anti-GGA1 was gift from Dr. Margaret S. Robinson (University of Cambridge, Cambridge, UK).

RNAi Experiment for Knockdowns—pSuper vectors of siRNA for GGA1–3 were obtained from Dr. Stuart Kornfeld (Washington University, St. Louis, MO) (22). HeLa cells were grown in Dulbecco’s modification of minimal essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the presence of 5% CO2. HeLa cells were cotransfected with pSuper GGA RNAi vectors and pSectag vector of memapsin 2 or mutants using transfection agent Lipofectamine 2000 (Invitrogen). The ratio of pSuper/RNAi plasmid DNA to pSectag was 4:1. 56 h after transfection, the cells were harvested for double-labeled immunofluorescence to detect colocalization of memapsin 2 with different organelle markers. siRNA knockdown of VPS26 was performed as described by Senman (23). siRNA oligonucleotides used to knock down VPS26 expression (AAUGAUGGGGAAACCAGGAAA) and control lamin A/C siRNA were obtained from Dharmacon. HeLa cells were cultured as described above in six-well dishes until 60–70% confluence. At least 4 h after seeding the cells, the first transfection was performed using Oligofectamine (Invitrogen). The cells were trypsinized the following day. Two transfections were performed, one at the start of the experiment and the other after 2 days. The cells were trypsinized 24 h after second transfection and were plated onto coverslips of six-well dishes for memapsin 2 transient overexpression and confocal microscopy observation.

Immunofluorescence Labeling of Cultured Cells—HeLa cells were seeded onto six-well plates with glass coverslips and expressed the memapsin 2 or mutant constructs for 56 h after transfection. Cells were gently fixed in 4% paraformaldehyde and in phosphate-buffered saline, pH 7.4, at room temperature for 15 min. Following fixation, coverslips were washed twice for 10 min in PBS and incubated for 1 h at room temperature with the indicated combinations of primary antibodies diluted in 0.1% bovine serum albumin, 0.1% saponin, and 0.02% sodium azide in PBS (immunofluorescence buffer). At the end of this period, coverslips were washed twice with PBS followed by incubation for 30 min with the indicated combinations of secondary antibodies diluted in immunofluorescence buffer. Following incubation, coverslips were washed again twice with PBS and mounted on slides using Vectorshield (Vector Laboratories, Inc., Burlingame, CA). Images were obtained in an inverted confocal laser-scanning microscope (LSM 510, Carl Zeiss Inc.). Human neuroblastoma M17 Cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the presence of 5% CO2. M17 cells were seeded onto six-well plates with glass coverslips. Endogenous memapsin 2 and GGA1 were observed as above.
Transient Transfection and Immunoprecipitation/Western Blotting—Plasmids containing separately memapsin 2 or its mutants and APPsw cDNAs were transiently transfected into HEK-293 cells in six-well plates using transfection reagent Lipofectamine 2000. Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (GibcoBRL). After transfection, cells were changed to fresh medium (1.5 ml for a six-well plate) conditioned for 48 h, subjected to immunoprecipitation and Western blotting.

Aβ peptides secreted into medium were immunoprecipitated at 4 °C overnight with monoclonal antibody mAb1560 (1:800) and protein G plus A-agarose (Sigma). Immunoprecipitates were washed for 20 min at 4 °C with lysis buffer (PBS with 1% Nonidet P-40 with protease inhibitors) and then washed in lysis buffer containing 0.1% SDS. The samples were washed again in lysis buffer, eluted in Laemmli sample buffer, separated
RESULTS

Subcellular Localization of Memapsin 2 and Mutants—We compared the intracellular distribution of wild-type memapsin 2 with that of two memapsin 2 mutants. One was a chimera, M2-MPR, in which the memapsin 2 cytosolic domain was replaced by the cytosolic domain of CD-MPR (Fig. 1A) that also contained an ACDL motif. Because GGA proteins (Fig. 1B) are known to be involved in the transport and retrieval between trans-Golgi and the early endosome (16, 17, 22, 24), this mutant serves for comparison with the localization and trafficking of memapsin 2. In the second mutant, M2-Leu/Ala, two leucines in the ACDL motif of memapsin 2 were replaced by alamines (10, 12), resulting in a mutant defective in endocytosis (10, 12) that served as a negative control. HeLa cells expressing memapsin 2 and mutants were visualized by immunocytochemical staining. The cellular localization pattern of wild-type memapsin 2 had a punctate distribution mainly in the perinuclear region (Fig. 1C), which confirmed previous reports (2, 9, 10) that the protease was localized primarily in TGN and early endosomes. Little memapsin 2 is seen on the cell surface. The colocalization of memapsin 2 with TGN and early endosome markers was confirmed by separate immunocytochemical staining with TGN38 (TGN marker) and EEA1 (results not shown). Immunolabeling pattern for cells expressing M2-MPR, which showed a similar perinuclear distribution as well as peripheral labeling (Fig. 1D), also colocalized well with TGN and early endosome markers (data not shown). The cellular distribution pattern of this chimera is similar to that of CD-MPR itself, which was found most abundantly in TGN, less in endosomes, and little on the cell surface (25). M2-Leu/Ala mutant showed a predominant localization on the cell surface (Fig. 1E) as reported previously (10, 12). These observations suggest that wild-type memapsin 2 is localized mainly in endosomes and trans-Golgi similar to MPR. The chimera of memapsin 2 with the cytosolic domain of CD-MPR also had an intracellular distribution similar to that of CD-MPR.

Memapsin 2 and GGA Proteins Colocalize in the Cell—Wild-type memapsin 2 and individual GGA proteins expressed in HeLa cells were examined using immunofluorescent staining and confocal microscopy. The distribution of GGA proteins in the cells was similar to that previously reported (19, 20). Extensive colocalization was seen for the wild-type memapsin 2 with each of the three GGA proteins in the perinuclear region, and some but much less extensive colocalization was observed in the peripheral structure (Fig. 2). Similar experiments were also performed on neuroblastoma M17 cells with the same results (Fig. 2, J–L). M2-MPR chimera also colocalized with each of the three GGA proteins with the distribution patterns similar to those observed for the wild-type memapsin 2 (Fig. 3). These observations suggest that MPR cytosolic domain in the chimera interacted with GGA proteins and directed the transport of M2-MPR to endosomes. However, memapsin 2 mutant M2-Leu/Ala was mainly present on the cell surface and did not significantly colocalize with any of the GGA proteins (Fig. 4). Very weak colocalization seen for GGA2 and GGA3 in the merged images may have resulted from residual interaction between M2-Leu/Ala and these two GGA proteins. These observations are consistent with the idea that all three GGA proteins function in the cellular transport of memapsin 2 in a manner similar to the transport of MPR (22).

Change in Memapsin 2 Intracellular Distribution in siRNA-Mediated Knockdown of GGA Proteins—To study further the involvement of GGA proteins in memapsin 2 intracellular trafficking, we determined the distribution of memapsin 2 in HeLa cells in which the synthesis of GGA proteins was silenced by using plasmid-based siRNA. Each of the three GGA proteins, but not the control protein, β-actin, was greatly diminished by the transfection of specific GGA siRNA vectors (Fig. 5). In mock-treated cells, the bulk of the wild-type memapsin 2 (Fig. 6B) and the memapsin 2-MPR chimera (Fig. 7B) was found in the juxtanuclear area as seen in the preceding figures. Silencing each GGA protein significantly increased the presence in the peripheral region of the cells and decreased at approximately the same level the juxtanuclear localization for both M2-WT (Fig. 6, E, H, and K) and M2-MPR (Fig. 7, E, H, and K). These changes are similar to those observed for cation-independent MPR (CI-MPR) when GGA proteins were individually depleted (22).

Immunostaining of these cells with different organelle markers revealed significant colocalization of M2-WT (Fig. 6, F, I, and L) and M2-MPR (Fig. 7, F, I, and L) with the early endosome marker EEA1 (only the colocalization with EEA1 is...
shown). Significantly, the EEA1 colocalization with peripheral memapsin 2 was substantially increased with GGA depletion, suggesting an increase of memapsin 2 in endosomes. In contrast, M2-Leu/Ala in HeLa cells exhibited no significant change from predominant cell surface localization upon the depletion of GGA proteins (data not shown). These observations suggest that all three GGA proteins participate in intracellular trafficking of native memapsin 2 and the M2-MPR chimera.

Change in Memapsin 2 Intracellular Distribution upon Mutation of Ser^{498} to Ala—We previously demonstrated that phosphorylation of Ser^{498} of memapsin2 within the ACDL motif increased the binding affinity of memapsin 2 for the VHS domain of GGA proteins (15). It was of interest then to determine whether the phosphorylated memapsin 2 ACDL was involved in the intracellular trafficking of the protease. We mutated Ser^{498} to an alanine to preclude phosphorylation at this position and compared the localization in cells expressing the wild-type and mutant memapsin 2. Immunofluorescence microscopy study showed that the mutation of Ser^{498} changed the distribution of memapsin 2 from perinuclear (Fig. 8, upper panels) to the peripheral localization (Fig. 8, lower panels). Consequently, the colocalization of the endosome marker EEA1 and the mutant M2-Ser/Ala was observed in the peripheral region but was not predominantly in the perinuclear region as seen for the wild-type memapsin 2. These observations are reminiscent of the changes found for GGA depletion discussed above and suggest that both GGA depletion and Ser^{498} mutation may block the same retrieval pathway for memapsin 2 from endosomes back to the cell surface.

Change of Memapsin 2 Intracellular Distribution in siRNA-mediated VPS26 Knockdown—The retrieval of MPR from endosomes back to Golgi has been shown to involve an intracellular protein complex called retromer (23). siRNA-mediated depletion of VPS26, a component protein of retromer, blocked the transport of CI-MPRs retrieval from endosome to Golgi. Because memapsin 2 and MPR both utilize ACDL/GGA interaction in cellular trafficking, it seemed possible that, in its recycling pathway, memapsin 2 is transported from endosomes to Golgi as an intermediate compartment before memapsin 2 reaches the cell surface. Therefore, we asked whether the depletion of VPS26 influenced the recycling of memapsin 2 from endosomes. siRNA-mediated depletion of VPS26 was nearly complete and did not affect the level of a control siRNA vector for β-actin (Fig. 9A). Images from immunochemical staining showed that the intracellular localization of memapsin 2 changed from a predominantly perinuclear localization with VPS26 expression (Fig. 9, B–D) to a peripheral localization (Fig. 9, E–G) upon VPS26 depletion. Some of the memapsin 2 was also found to be present at the plasma membrane. This may have resulted from the backup of the trafficking due to serious disruption of endosome to Golgi transport. However, in general, the similarity in intracellular distribution changes with VPS26 depletion, GGA depletion, or Ser^{498} mutation is striking. Such a comparison, together with the known role of the retromer in MPR retrieval, suggests that memapsin 2 recycles from endosomes by way of the Golgi network.

APP Hydrolysis and Aβ Production Mediated by Memapsin 2 Mutants—Because alterations in memapsin 2 distribution and trafficking were observed in the experiments described above, it was of interest to assess the effect of these changes on APP hydrolysis by memapsin 2. Therefore, we compared the memapsin 2-generated APP C-terminal fragment C99 in HEK-293 cells cotransfected with APPsw and each of the following: M2-WT; M2-MPR; M2-Leu/Ala, and M2-Ser/Ala. 48 h after transfection, the expression of memapsin 2 and APP was confirmed by Western blots (Fig. 10, A and B). Chimera M2-MPR and mutants M2-Leu/Ala and M2-Ser/Ala each produced APP fragment C99 (Fig. 10C) and Aβ (Fig. 10D) at a comparable level as that produced by the wild-type memapsin 2. The immunoprecipitated soluble APP fragment resulting from β-secretase cleavage was seen in M2-WT as well as in M2-MPR, M2-Leu/Ala, and M2-Ser/Ala experiments (data not shown). These ob-
The cytosolic domain of memapsin 2 was probably phosphorylated and interacted with GGA proteins to facilitate its transport from endosomes via TGN to cell surface.

We previously demonstrated the interaction between GGA proteins and the native and phosphorylated ACDL motif in the cytosolic domain of memapsin 2 (14, 15). These observations and the knowledge that a similar interaction of GGA proteins with MPR mediates the transport of MPR between the trans-Golgi and endosomes prompted the suggestion that GGA proteins were involved in the intracellular transport of memapsin 2. However, the supporting cellular studies had not been carried out and the specific point of GGA involvement in memapsin 2 trafficking was unknown. GGA proteins are found predominantly in the TGN but are also present in early endosomes (19, 20). We observed cellular colocalization of GGA proteins with memapsin 2 and alteration of the memapsin 2 intracellular locations upon siRNA-mediated GGA depletion. These observations support the contention that GGA proteins are involved in the cellular transport of memapsin 2. As observed in MPR transport (22), all three GGA proteins are involved in the intracellular trafficking of memapsin 2 because the depletion of any of the three GGA proteins caused a significant redistribution of memapsin 2. In the transport of CI-MPR, three GGA proteins are interdependent in their functions (22). Although we did not provide evidence for this aspect in memapsin 2 transport, it seems probable that the mechanism of involvement of GGA in the transport of the two MPRs and memapsin 2 is the same, namely the recognition of the ACDL motif facilitates packaging the proteins into the transporting vesicles. Our results also support the contention that GGA proteins interact with the phosphorylated ACDL motif of memapsin 2 in the recycling pathway from endosomes to TGN. It is also interesting that the ACDL mutated to preclude phosphorylation resulted in a redistribution of cellular memapsin 2 similarly as observed upon depletion of GGA proteins. This similarity suggests that the peripherally localized memapsin 2 resulting from blockage is achieved by siRNA or by mutation of ACDL. The similarity of the redistribution of memapsin 2 and CI-MPR affected by siRNA for GGA proteins is also striking. The routes for memapsin 2 and CI-MPR to reach endosomes are presumably different. CI-MPR is predominantly transported to the endosome from the Golgi, and memapsin 2 is known to be endocytosed to endosomes from cell surface. The similarity can be explained from the GGA involvement in the recycling pathway of both proteins, i.e. the depletion of GGA would result in the accumulation of both proteins in endosomes.

In its recycling pathway, memapsin 2 is probably transported from endosomes to TGN as an intermediate stop before returning to cell surface by the usual secretory pathway. Recent evidence suggests that retromer protein complex is specifically involved in the endosome to TGN transport of MPR (23). Depletion of VPS26, a subunit of retromer complex, caused the redistribution of memapsin 2 from predominantly perinuclear to peripherally localized endosomes. These observations are consistent with the contention that the loss of retromer functions blocks the trafficking from endosomes to TGN and thus causes accumulation of memapsin 2 in the endosomes. However, we did observe the accumulation of memapsin 2 at the cell surface upon retromer depletion. It is not clear whether this is due to the backup of the memapsin 2 traffic or the involvement of retromer in other trafficking routes of memapsin 2. However is the cause, the complication does not appear to change the main conclusion that memapsin 2 recycles through TGN. The overall evidence discussed above is consistent with the notion that phosphorylated memapsin 2 is recycled via TGN on its way back to cell surface. However, identification of specific observations suggest that both memapsin 2 mutants hydrolyzed APP intracellularly to generate Aβ.

**FIG. 9. Depletion of VPS26 affects the intracellular distribution of memapsin 2.** A, specific depletion of VPS26. HeLa cells were transfected twice with either control siRNA (lamin A/C) or siRNA VPS26 within 72 h. After harvest of cells, equal aliquots of cell extracts were subjected to SDS-PAGE and Western blotting with appropriate antibodies to evaluate the efficiency of knockdown achieved. B–G, depletion of VPS26 affects the localization of memapsin 2. HeLa cells transfected with either control siRNA (B–D) or siRNA VPS26 (E–G) were transfected with M2-WT, immunostained for memapsin 2 (red) and EEA1 (green), and examined by confocal fluorescence microscopy.

**FIG. 10. Western blot analysis of APP fragments in HEK-293 cells transfected with APPsw and different memapsin 2 constructs.** Each experiment was repeated at least three times, and the representative data are shown here. A, memapsin 2 or mutants were overexpressed in 293 cells. B, the representative Western blot of full-length APPsw overexpressed in 293 cells. C, APP C-terminal fragments in HEK-293 cell lysates. Cells were lysed in 1% Nonidet P-40 buffer 48 h after transfection, and proteins were separated on 10–20% Tricine gels, in HEK-293 cell lysates. Cells were lysed in 1% Nonidet P-40 buffer 48 h after transfection, and proteins were separated on 10–20% Tricine gels, transferred to PVDF, and blotted with the AB5352. D, total Aβ from conditioned medium of various transfected HEK-293 cells was immunoprecipitated with mAb1560, separated on 10–20% Tricine gel, transferred to PVDF, and blotted with mAb1560. Note that some Aβ in the first lane is the result of leakage from the second lane.

**FIG. 11.** Western blot analysis of APP fragments in HEK-293 cells transfected with APPsw and different memapsin 2 constructs. Each experiment was repeated at least three times, and the representative data are shown here. A, memapsin 2 or mutants were overexpressed in 293 cells. B, the representative Western blot of full-length APPsw overexpressed in 293 cells. C, APP C-terminal fragments in HEK-293 cell lysates. Cells were lysed in 1% Nonidet P-40 buffer 48 h after transfection, and proteins were separated on 10–20% Tricine gels, transferred to PVDF, and blotted with the AB5352. D, total Aβ from conditioned medium of various transfected HEK-293 cells was immunoprecipitated with mAb1560, separated on 10–20% Tricine gel, transferred to PVDF, and blotted with mAb1560. Note that some Aβ in the first lane is the result of leakage from the second lane.

**DISCUSSION**

Evidence reported in this study and previous reports quoted herein suggest an intracellular transport route of memapsin 2. Observation suggests that both memapsin 2 mutants hydrolyzed APP intracellularly to generate Aβ.
subcellular compartments along this recycling route would require detailed studies in the future.

Current evidence does not support the involvement of GGA proteins in the endocytosis of memapsin 2. We did not observe significant colocalization of GGA and memapsin 2 at the cell surface (Fig. 2). The depletion of GGA proteins did not cause a significant accumulation of memapsin 2 on cell surface (Fig. 6). Because the cytosolic GGA proteins should be accessible for memapsin 2 located both in endosomes and at cell surface, the specificity of recycling involvement may be determined by the phosphorylation of the memapsin 2 ACDL motif. We have shown that the phosphorylated memapsin 2 ACDL binds GGA proteins with ~20 times lower $K_d$ than the non-phosphorylated ACDL. This tighter binding may be sufficient to provide specificity for the memapsin 2/GGA interaction. If such a mechanism was operative, it would predict the presence of a memapsin 2 ACDL kinase in the endosomes and a phosphatase in the TGN or beyond.

The above discussion raises another interesting question regarding the identity of the endocytic signal for memapsin 2 endocytosis. We have confirmed the previous report (10, 12) that two leucines within the ACDL motif appear to be essential for memapsin 2 endocytosis, because the replacement of these residues with alanines resulted in the accumulation of the mutant protease on cell surface (Fig. 4). Dileucine is a well established endocytotic signal (26), and in this case, it appears to function independent of the GGA/ACDL interaction. Another possible internalization route is the APP-dependent endocytosis. We reported recently (21) that the exogenously added memapsin 2 ectodomain is endocytosed by way of an interaction with APP. This process is dependent on the endocytic signal GYENPTY in the cytosolic domain of APP. Full-length memapsin 2 appears to also interact with APP, suggesting that the APP-dependent endocytosis of memapsin 2 may be a viable pathway.

In all of the experiments describe herein, M2-MPR chimera responded to all different challenges in almost the same way as the native memapsin 2. Superficially, this suggests that the cytosolic domain of MPR may contain the same targeting information as memapsin 2. The comparison of the two in the current studies was restricted to the recycling pathway that the two native proteins share. However, the pathway leading to endosomes should be different for these two proteins, although it is known that a small fraction of MPR is endocytosed from cell surface.

We observed that all of the memapsin 2 constructs, including M2-MPR chimera, produced $\alpha$-secretase and the C99, the C-terminal fragment of APP (Fig. 10). However, the subcellular locales where the cleavage takes place are difficult to dissect. Although the main subcellular site for the generation of $\alpha$-secretase is endosomes, the production of a significant amount of $\alpha$-secretase in the secretory pathway has been documented (27–29). In the case of the dileucine to alanine mutant of memapsin 2 (M2-Leu/Ala in Fig. 10), the $\alpha$-secretase generated should predominantly come from the...
secretory pathway because this mutant is devoid of endocytic ability. In the case of memapsin 2-MPR chimera (M2-CD in Fig. 10), the presence of C89 (Fig. 10), an APP fragment from the cleavage by cell surface α-secretase, suggests that M2-MPR chimera is capable of reaching cell surface and experiences cleavage by cell surface 10), the presence of C83 (Fig. 10), an APP fragment from the ability. In the case of memapsin 2-MPR chimera (M2-CD in Fig. 10) mutant, and perhaps the cell surface exposure of this protein is a transient one.

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REFERENCES
1. Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A., and Tang, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1456–1460
2. Vassar, R., Bennett, B. D., Babu-khan, S., Mendiaz, R. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jaroninski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
3. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brushier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Nature 402, 533–537
4. Hussain, J., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) Mol. Cell. Neurosci. 14, 419–427
5. Selkoe, D. (2001) Physiol. Rev. 81, 741–766
6. Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A., Zhang, X. C., and Tang, J. (2000) Science 290, 150–153
7. Bennett, B. D., Denis, P., Haniu, R., Teplow, D. B., Kahn, S., Louis, J. C., Citron, M., and Vassar, R. (2000) J. Biol. Chem. 275, 37712–37717
8. Creemers, J. W., Dominguez, D. I., Piets, E., Serneels, L., Taylor, N. A., Multarg, G., Craessaerts, K., Annaert, W., and De Strooper, B. (2000) J. Biol. Chem. 276, 4211–4217
9. Capell, A., Steinier, H., Willem, M., Kaiser, H., Meyer, C., Walter, J., Lammisch, S., Multhaup, G., and Haass, C. (2000) J. Biol. Chem. 275, 30849–30854
10. Huse, J. T., Pijak, D. S., Leslie, G. J., Lee, V. M.-Y., and Doms, R. W. (2000) J. Biol. Chem. 275, 33729–33737
11. Walter, J., Fluherr, R., Hartung, B., Willem, M., Kaether, C., Capell, A., Lammisch, S., Multhaup, G., and Haass, C. (2001) J. Biol. Chem. 276, 14654–14661
12. Fastorino, L., Ibin, A. F., Narin, A. C., Pursnani, A., and Buxbaum, J. D. (2002) Mol. Cell. Neurosci. 19, 175–185
13. Bonifacino, J. S. (2004) Nat. Rev. Mol. Cell. Biol. 5, 25–32
14. He, X., Chang, W. P., Koelsch, G., and Tang, J. (2002) FEBS Lett. 524, 183–187
15. He, X., Zhu, G., Koelsch, G., Rodgers, K. K., Zhang, X. C., and Tang, J. (2003) Biochemistry 42, 12174–12180
16. Puertollano, R., Aguilar, R. C., Gorshkova, I., Crouch, R. J., and Bonifacino, J. S. (2001) Science 292, 1712–1716
17. Zhu, Y., Doray, B., Poussa, A., Lehto, V. T., and Kornfeld, S. (2001) Science 292, 1716–1718
18. Takatsu, H., Kato, Y., Shiba, Y., and Nakayama, K. (2001) J. Biol. Chem. 276, 28541–28545
19. Roman, A. L., Zhang, C., Zhu, X., and Kahn, R. A. (2000) Mol. Cell. Biol. 11, 1241–1255
20. Hirst, J., Lui, W. W., Bright, N. A., Totty, N., Seamen, M. N., and Robinson, M. S. (2000) J. Cell Biol. 149, 67–80
21. Huang, X. P., Chang, W. P., Koelsch, G., Turner, R. III, Lupu, F., and Tang, J. (2004) J. Biol. Chem. 279, 37886–37894
22. Ghosh, P., Griffith, J., Geuze, H. J., and Kornfeld, S. (2003) Annu. Rev. Biochem. 72, 395–447
23. Senman, M. N. J. (2004) J. Cell Biol. 163, 755–766
24. Puertollano, R., Aguilar, R. C., Gorshkova, I., Crouch, R. J., and Bonifacino, J. S. (2001) Science 292, 1712–1716
25. Mathews, P. M., Guerra, C. B., Jiang, Y., Grbovic, O. M., Kao, B. H., Schmidt, S. D., Dinakar, R., Mercken, M., Hille-Rehfeld, A., Rohrer, J., Mehta, P., Cataldo, A. M., and Nixon, R. A. (2002) J. Biol. Chem. 277, 5299–5307
26. Bonifacino, J. S., and Traub, L. M. (2003) Annu. Rev. Biochem. 72, 395–447
27. Citron, M., Taplow, D. B., and Selkoe, D. J. (1995) Neuron 14, 661–670
28. Huse, J. T., Liu, K., Pijak, D. S., Carlin, D., Lee, V. M.-Y., and Doms, R. W. (2002) J. Biol. Chem. 277, 16278–16284
29. Steinhilb, M. L., Turner, R. S., and Gart, J. R. (2002) J. Neurochem. 80, 1019–1028
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Xiangyuan He, Feng Li, Wan-Pin Chang and Jordan Tang

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