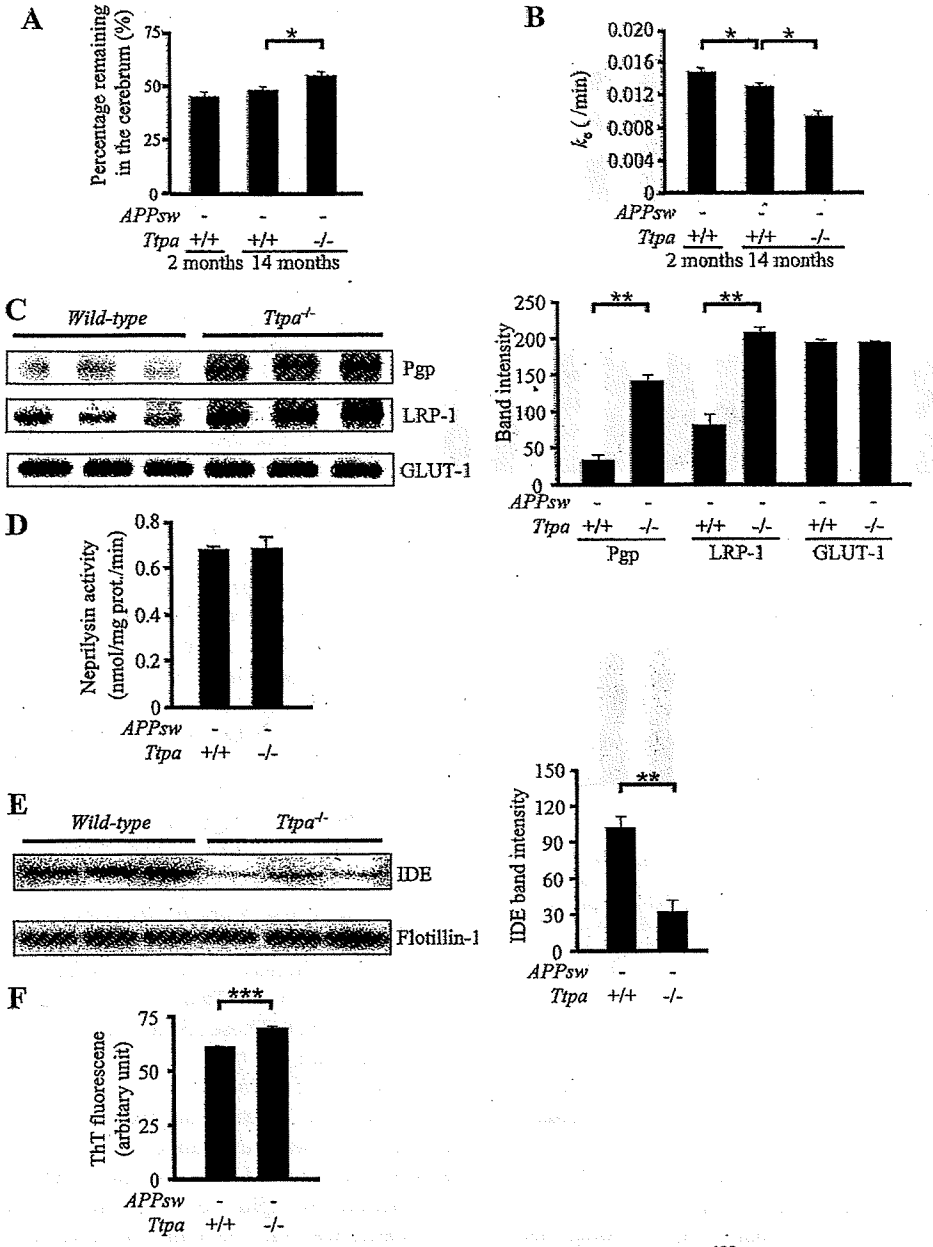

FIGURE 3. **α-Toc depletion decreases Aβ clearance.** *A*, the remaining percentage of ^{125}I-Aβ_{1-40} at 60 min was higher in 14-month-old *Ttpa*$^{-/-}$ mice than in age-matched wild-type mice. *B*, k_e was markedly decreased in *Ttpa*$^{-/-}$ mice. *C*, protein levels of LRP-1 and Pgp were increased in the brains of *Ttpa*$^{-/-}$ mice compared with wild-type mice, whereas levels of GLUT-1 were not changed between them. Band intensities are shown in the *right panel*. *D*, neprilysin-dependent endopeptidase activity did not decrease in *Ttpa*$^{-/-}$ mice compared with wild-type mice. *E*, protein levels of IDE were decreased in the brains of *Ttpa*$^{-/-}$ mice compared with wild-type mice. Band intensities are shown in the *right panel*. *F*, thioflavin T (*ThT*) fluorescence intensity in the incubation mixtures of synaptosomes with synthetic Aβ_{1-40} was increased in the brains of *Ttpa*$^{-/-}$ mice compared with wild-type mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

much greater than that by aging, as shown between 2 and 14 months of age (13.4%). One of the most likely pathologies influencing Aβ clearance is the compromised BBB by oxidative stress with vitamin E deficiency. However, there is no evidence of abnormal structures of endothelial cells or ischemic change on histological analysis of the *Ttpa*$^{-/-}$ mouse brains (data not shown).

Efflux Transporters for Aβ across the BBB Are Up-regulated in the Brain Capillary Endothelial Cells of Ttpa$^{-/-}$ *Mouse*—One of possible causes of impaired Aβ clearance is the decreased efflux and the decreased degradation of Aβ. To examine whether reported molecules involved in Aβ transport at the BBB were down-regulated, we measured the protein levels of LRP-1 and Pgp. Surprisingly, both protein levels in the small vascular fraction in the brains of *Ttpa*$^{-/-}$ mice were much increased compared with wild-type mice (Fig. 3*C*). In contrast, there was no change in the levels of GLUT-1, which is a transporter localized to the brain capillary endothelial cells (27).

Aβ-degrading Peptidase, IDE, Is Decreased in Ttpa$^{-/-}$ *Mouse Brain*—Next, we studied Aβ-degrading peptidases, neprilysin and IDE, for studying another possible cause of impaired Aβ clearance. Although the enzymatic activity of neprilysin was not decreased in *Ttpa*$^{-/-}$ mouse brain compared with wild-type mouse (Fig. 3*D*), expression level of IDE was markedly decreased in *Ttpa*$^{-/-}$ mouse brain (Fig. 3*E*). We, therefore, consider that an impaired degradation of Aβ in the brain because of decreased IDE is related with enhanced Aβ accumulation in *Ttpa*$^{-/-}$*APPsw* mouse brain.

Aβ Aggregation Is Accelerated in Ttpa$^{-/-}$ *Mouse Brains*—Moreover, we studied the effect of oxidative stress on Aβ aggregation capacity. The aggregation of Aβ_{1-40} in the presence of synaptosomes was measured by using thioflavin T fluorescence. The aggregation capacity was increased in brain homogenates of the *Ttpa*$^{-/-}$ mice compared with wild-type homogenates (Fig. 3*F*).

Ttpa$^{-/-}$*APPsw Mouse Has an Increased Level of Aβ in the Plasma as Well as in the Brain*—Furthermore, we measured the plasma levels of Aβ in *Ttpa*$^{-/-}$*APPsw* mouse. The 18-month-old *Ttpa*$^{-/-}$*APPsw* mice showed markedly increased levels of both plasma Aβ_{1-40} and Aβ_{1-42} (Fig. 4*A*). These accumulations of Aβ_{1-40} and Aβ_{1-42} were partially recovered when *Ttpa*$^{-/-}$*APPsw* mice were fed on the α-Toc-supplemented diet (Fig. 4*A*). In contrast, the Aβ-binding proteins in the plasma,

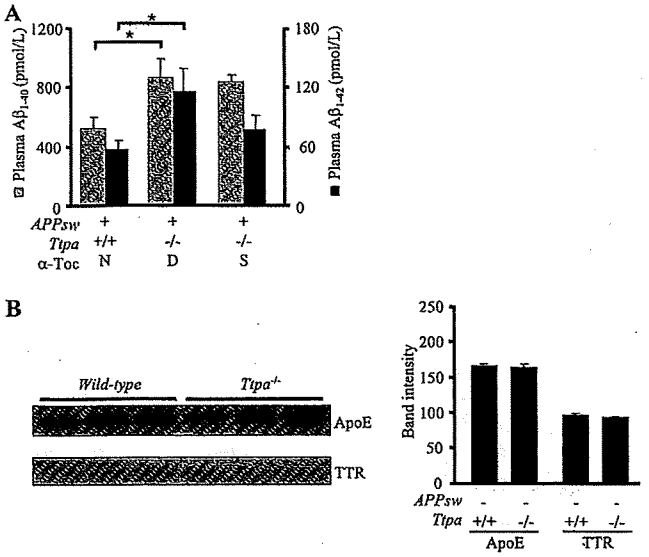

FIGURE 4. **The *Ttpa*$^{-/-}$*APPsw* mouse shows enhanced accumulation of Aβ in the plasma.** *A*, 18-month-old *Ttpa*$^{-/-}$*APPsw* mice showed increased levels of $A\beta_{1-40}$ and $A\beta_{1-42}$. This increase was partially ameliorated by α-Toc supplementation in the diet. *B*, protein levels of apoE and TTR were not changed in the plasma of *Ttpa*$^{-/-}$ mice compared with wild-type mice. Band intensities are shown in the *right panel*. *D*, α-Toc-deficient diet; *S*, α-Toc-supplemented diet; *N*, normal diet. *, $p < 0.05$.

apoE, and TTR levels were not different between *Ttpa*$^{-/-}$ mice and wild-type mice (Fig. 4*B*).

Increased Aβ Accumulation in the Plasma Is also Caused by Impairment of Aβ Clearance from the Blood—The systemic clearance of Aβ should influence the levels of plasma Aβ. Therefore, the effect of *Ttpa* deficiency on systemic clearance of Aβ from the circulation was investigated *in vivo*. We also used *Ttpa*$^{-/-}$ in this experiment instead of *Ttpa*$^{-/-}$*APPsw* and *APPsw* mice, because the CLtot, a primary pharmacokinetic parameter that is a measure of the elimination efficiency of peripherally injected ^{125}I-$A\beta_{1-40}$, is known to decrease significantly in the presence of high plasma levels of $A\beta_{1-40}$ (28). Fig. 5*A* shows the plasma concentration-time profiles of trichloroacetic acid-precipitable ^{125}I-$A\beta_{1-40}$ after intravenous bolus administration in 2- and 25-month-old wild-type and 25-month-old *Ttpa*$^{-/-}$ mice. Plasma concentration of trichloroacetic acid-precipitable ^{125}I-$A\beta_{1-40}$ in 25-month-old *Ttpa*$^{-/-}$ mice was significantly greater at 1, 3, 60, and 360 min and substantially greater at all time points than that in 25-month-old wild-type mice (supplemental Table 1). As shown in supplemental Table 2, the AUC for *Ttpa*$^{-/-}$ mice was significantly greater than that for age-matched wild-type mice by 2.8-fold. To evaluate the systemic clearance more in detail, other pharmacokinetic parameters were determined and summarized in supplemental Table 2. In 25-month-old *Ttpa*$^{-/-}$ mice, CLtot and k_e (elimination rate constant) were significantly decreased by 41.2 and 51.7%, respectively, compared with those in age-matched wild-type mice (Fig. 5*B* and supplemental Table 2). The reduction in CLtot evoked by α-Toc depletion (41.2%) was much greater than that by aging, as shown between 2 and 25 months of age (14.1%) in model-independent moment analysis. The similar results were obtained in model dependent analysis as well (supplemental Table 2).

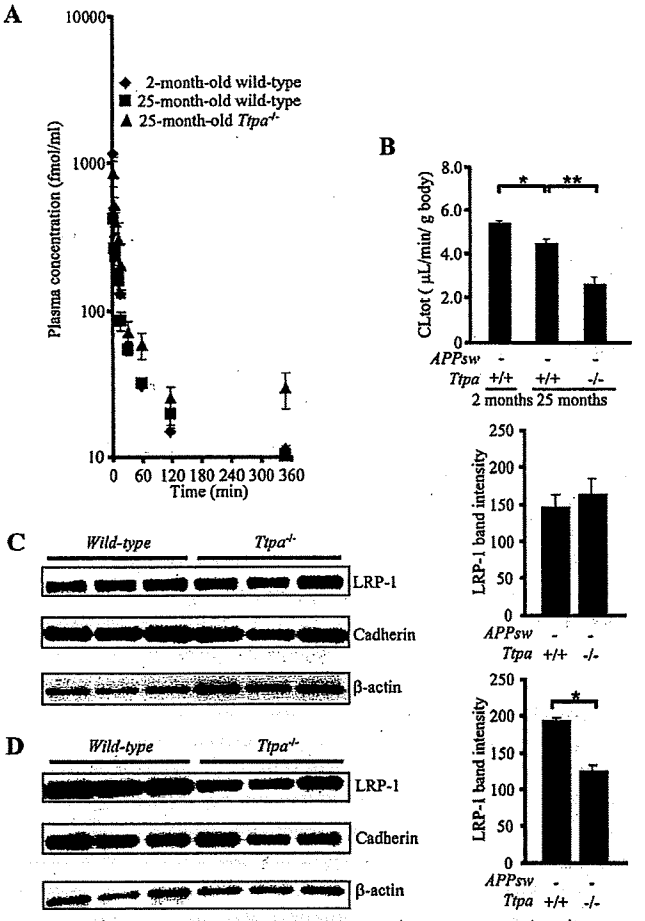

FIGURE 5. **α-Toc depletion decreases Aβ clearance from the plasma.** *A*, the remaining level of trichloroacetic acid-precipitable ^{125}I-$A\beta_{1-40}$ after its injection from the jugular vein was higher in 25-month-old *Ttpa*$^{-/-}$ mice than in wild-type mice. *B*, the total body clearance of ^{125}I-$A\beta_{1-40}$ was markedly decreased in *Ttpa*$^{-/-}$ mice compared with wild-type mice. *C* and *D*, the protein level of LRP-1 was decreased not in the crude membrane fraction of the liver of *Ttpa*$^{-/-}$ mice (*C*) but in the plasma membrane fraction (*D*) compared with wild-type mice. *, $p < 0.05$.

These results demonstrated that systemic clearance was attenuated in 25-month-old *Ttpa*$^{-/-}$ mice, and the decrease in the systemic clearance is likely to be because of a decrease in the clearance from the liver, as the systemic clearance of Aβ has been reported to be mostly mediated by clearance from the liver (29, 30).

LRP-1 Is Down-regulated in the Plasma Membrane Fraction of the Liver in Ttpa$^{-/-}$ *Mice*—To examine whether the Aβ receptor was down-regulated in the liver for the cause of impaired Aβ clearance from the blood, we measured the protein level of LRP-1 in the crude and plasma membrane fractions of the liver, as LRP-1 translocates from Golgi apparatus to plasma membrane in their activation for transporting Aβ into the hepatocytes (31). The protein level of LRP-1 was unchanged in the crude membrane fraction but decreased in the plasma membrane fraction of *Ttpa*$^{-/-}$ mouse liver (Fig. 5, *C* and *D*). This inactivation of LRP-1 might explain the decreased clearance of Aβ from the blood, causing increased Aβ in *Ttpa*$^{-/-}$*APPsw* mouse plasma.

DISCUSSION

We clearly demonstrated that Aβ clearances from the brain and from the blood were decreased in *Ttpa*$^{-/-}$ mice. Because the Aβ generation in *Ttpa*$^{-/-}$*APPsw* mouse brain was not increased, we consider that accumulated Aβ in *Ttpa*$^{-/-}$*APPsw* mouse brain is caused by these impaired Aβ clearances. The Aβ clearance from the brain can be accomplished via two major pathways; that is, receptor-mediated transport from the brain and proteolytic degradation in the brain. First, two proteins expressed in brain endothelial cells, LRP-1 and Pgp, are reported to regulate Aβ clearance by controlling its efflux from brain to blood based on the studies of genetically engineered mice (32, 33). Actually, LRP-1 was down-regulated in older mice, and this down-regulation correlated with Aβ accumulation in AD brains (25). Pgp expression was also inversely correlated with deposition of Aβ in the brains of elderly non-demented humans (34). Surprisingly, both LRP-1 and Pgp levels are markedly increased in *Ttpa*$^{-/-}$ mouse brains, although clearance of ^{125}I-Aβ$_{1-40}$ by the BEI method is impaired. There are two possible explanations for the up-regulations of LRP-1 and Pgp. One is to compensate their dysfunctions, and another is to transport increased other substrates in the brain caused by lipid peroxidation. Second, the two major endopeptidases involved in proteolysis-related degradation of Aβ in the brain are neprilysin and IDE. Whereas the activity of neprilysin was not decreased, the protein level of IDE was markedly decreased in *Ttpa*$^{-/-}$ mouse brain. Furthermore, we made a gene chip analysis and evaluated all the molecules cyclopedically in the brains of *Ttpa*$^{-/-}$ and wild-type littermate mice. As a result, the only reasonable change of expression level for possibly causing enhanced Aβ accumulation was the decrease in IDE mRNA (supplemental Table 3, *A* and *B*). The homozygous deletion of IDE gene are known to show decreased Aβ degradation and increased accumulation of endogenous Aβ in the mouse brains (35, 36). Moreover, we previously confirmed that contribution of IDE to the clearance of microinjected ^{125}I-Aβ$_{1-40}$ in the BEI method could be 25.3% by the pre-administration of IDE inhibitors, bacitracin (26). Together, as one of molecular mechanisms of Aβ accumulation and impaired clearance of Aβ in *Ttpa*$^{-/-}$*APPsw* mouse brains, we think that degradation of Aβ was impaired by decreased expression of IDE. However, we cannot exclude the possibility of dysfunction of other proteins because of lipid peroxidation, which may contribute to abnormal Aβ metabolism.

There are reports that AD patients showed increased levels of peripherally circulating Aβ (37, 38). In *Ttpa*$^{-/-}$*APPsw* mice as well, plasma Aβ levels are proved to be markedly increased to be compared with *APPsw* mice. Significantly lowered clearance of injected ^{125}I-Aβ$_{1-40}$ from the *Ttpa*$^{-/-}$ mouse blood could explain the increased plasma Aβ in *Ttpa*$^{-/-}$*APPsw* mice. Although the excretion of Aβ through the kidney accounts only for a minute portion of Aβ in the blood (39), the liver is the major organ responsible for blood clearance of Aβ (29). We previously reported that LRP-1 in hepatocytes plays an important role to uptake plasma Aβ because mice with down-regulated LRP-1 by knock-out of receptor-associated protein or hydrodynamic injection of siRNA showed a much decreased uptake of ^{125}I-Aβ$_{1-40}$ into the liver (40). The 85-kDa LRP-1 is proteolytically cleaved from a 600-kDa precursor in Golgi apparatus and is translocated by receptor-associated protein to plasma membrane to be activated for bounding Aβ (41). The result of decreased LRP-1 in the plasma membrane fraction without change of LRP-1 level in the crude membrane fraction of *Ttpa*$^{-/-}$ mouse liver indicated that lipid peroxidation does not affect LRP-1 expression itself but suggested a disturbed translocation of LRP-1. In another view, plasma Aβ level could be influenced by a change of its plasma ligands; apoE, TTR, and soluble LRP-1 (30, 42, 43). When soluble LRP-1 is oxidized, it is known to be decreased in its affinity to Aβ$_{1-40}$ and Aβ$_{1-42}$ (43). Although serum apoE and TTR levels in *Ttpa*$^{-/-}$ mice were not decreased, we could not evaluate serum soluble LRP-1 nor oxidized soluble LRP-1 level. Therefore, we cannot exclude the possibility that soluble LRP-1 is affected by the lipid peroxidation and causes the increased Aβ accumulation in *Ttpa*$^{-/-}$*APPsw* mouse plasma.

Given the fact that a large number of sporadic AD cannot be explained by increased Aβ generation (3), better understanding of the molecular and genetic basis of the Aβ clearance mechanisms may hold at least in part the key for research of AD pathology. A strongest risk factor for AD is aging (44), and lipid peroxidation may be a major cause for aging of the brain (45). In these respects, our findings of increased accumulation and aggregation of Aβ with impaired clearance due to lipid peroxidation are new aspects of AD pathology. We hope that further investigation on the molecular mechanism of impaired Aβ clearance due to lipid peroxidation provides a novel diagnostic and therapeutic target of AD.

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Visual screening and analysis for kinase-regulated membrane trafficking pathways that are involved in extensive β-amyloid secretion

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Membrane trafficking is an important cellular process that enables the precise localization of membrane proteins. The disturbance of membrane trafficking results in various disease states. To explore systematically the defects in trafficking pathways that cause these disturbances or disease states, we developed an automated high-throughput fluorescence-based imaging system and carried out visual screening for kinase-regulated trafficking pathways of the cation-independent mannose 6-phosphate receptor (CI-M6PR) in HeLa cells. As the result of our visual screening, which examined the effect of kinase inhibitors and a kinase siRNA library, we identified five kinases (CDC42BPB, PRKACA, PRKACG, GSK3β and CSNK2A1) that regulate CI-M6PR trafficking. Moreover, we focused on Alzheimer's disease (AD) to study the relationship between the five kinases and a disease state. Notably, two trafficking pathways, which were regulated by PRKACG and GSK3β, respectively, induced high levels of secretion of Aβ, the hallmark of AD. In addition, we found that the modulation of GSK3β activity affected the microtubule plus end tracking function of cytoplasmic linker protein-associating protein 2 and resulted in the perturbation of BACE1 localization/trafficking and extensive Aβ secretion. Our systems provide new approaches for the analysis of spatially-regulated membrane trafficking and related disease states.

Introduction

Disorders of membrane trafficking pathways cause the disruption of a various cellular functions and sometimes lead to cell death. However, there are few available analytical approaches that allow the investigation of the causal connections between a disrupted trafficking pathway and the pathogenesis of a disease that arises from this disruption. Localizomics is a new field that provides information about the localization of proteins and lipids, and allows the elucidation of the mechanisms that regulate these processes. Our aim was first to establish a versatile functional screening and analytical system for identifying kinases that regulate the membrane trafficking pathways of specific membrane proteins by using fluorescence image-based visual screening techniques. We would then use this system to elucidate the causal connections between a defective transport pathway and the pathogenic mechanisms of diseases that might be caused by mislocalization of the protein. Protein kinases are the most versatile among the many regulators of membrane trafficking. The kinase network has been studied extensively and kinases are likely to prove powerful tools in the search for upstream or downstream proteins that regulate the trafficking of membrane proteins.

To this end, we focused on the membrane trafficking of the cation-independent mannose 6-phosphate receptor (CI-M6PR) and the causal connections between its mislocalization and Alzheimer's disease (AD). CI-M6PR is involved in the trafficking of a broad range of lysosomal enzymes from the trans-Golgi network (TGN) or the cell surface to lysosomes; it mainly shuttles between endosomes and the TGN (Ghosh *et al.* 2003; Arighi *et al.* 2004; Scott *et al.* 2006). It has been shown that defects in the shuttling of CI-M6PR between endosomes and the TGN result in the perturbation of its localization in the cell and can cause many pathogenic states; for example,

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Niemann-Pick disease, I-cell disease, and, in particular, AD (Cataldo *et al.* 1997; Mathews *et al.* 2002; Tomiyama *et al.* 2004; Kar *et al.* 2006; Waguri *et al.* 2006). One of the principal pathological hallmarks of AD is the deposition of amyloid β (Aβ), in the form of senile plaques, throughout the hippocampus and neocortex. Aβ is derived from the amyloid precursor protein (APP), and is formed by the sequential cleavage of APP by the β- and γ-secretase enzymes (Saido & Iwata 2006). The localization of β-site APP-cleaving enzyme 1 (BACE1), which is a transmembrane protease with β-secretase activity, is regulated by an acidic-cluster di-leucine motif, which is recognized by Golgi-localized, γ-ear-containing, ADP-ribosylation factor binding protein (Shiba *et al.* 2004; He *et al.* 2005; Tesco *et al.* 2007). Golgi-localized, γ-ear-containing, ADP-ribosylation factor binding protein also plays a crucial role in the trafficking of CI-M6PR. In addition, knockdown by RNA interference (RNAi) of the vacuolar protein sorting protein VPS26, which is another regulator of CI-M6PR transport, has been reported to induce an increased secretion of Aβ40 (Small *et al.* 2005). Therefore, we predicted that kinases that perturb the vesicular transport of CI-M6PR between the endosomes and the TGN would modulate the extent of Aβ secretion.

In this article, we have used cell array chips and gene silencing by RNAi, in combination with visual assays, to establish a two-step functional screening system for human kinases, or their regulators, that might be involved in the localization or shuttling of CI-M6PR. Using this method, we identified five candidate kinases. Furthermore, we found that knockdown of PRKACG (protein kinase, cAMP-dependent, catalytic, gamma) or glycogen synthase kinase 3β (GSK3β), which were two of the five candidates, induced the extensive production of secreted Aβ, and concurrently perturbed the intracellular distribution of BACE1. These results indicated that decreased activity of these two kinases is a possible risk factor for AD. In addition, we found that a functional defect in cytoplasmic linker protein-associating protein 2 (CLASP2), which arose due to the modulation of GSK3β activity, perturbed membrane trafficking between the endosomes and the TGN, and resulted in the mislocalization of BACE1 and extensive Aβ secretion.

Results

Strategy for clarifying the causal connections between the localization of a given protein and the pathogenic mechanism of a related disease

The scheme for our screening and analysis is shown in Fig. 1. To identify human kinases that might be involved in the localization or shuttling of CI-M6PR, we used fluorescence imaging to monitor changes in the localization of CI-M6PR. Immunofluorescence analysis showed an extensive co-localization of CI-M6PR with p230, which is a marker of the TGN, under normal conditions in HeLa cells (Fig. 1A, control). However, if shuttling is perturbed using an appropriate kinase inhibitor or an siRNA against a particular kinase, CI-M6PR that is being recycled will be trapped in the early/late endosomes or at the plasma membrane (PM). As a result, the fluorescence signal from CI-M6PR becomes dispersed throughout the cytoplasmic endosomes and no longer overlaps with that of p230. The morphology of the Golgi, however, is virtually unaffected, as shown by the staining pattern of p230. For example, treatment of HeLa cells with 3-methyladenine (3-MA), which is a specific inhibitor of the class III phosphoinositide 3-kinase (PI3K) and is known to inhibit retrograde transport from early endosomes to the TGN, resulted in a dispersed signal for CI-M6PR that did not co-localize with that of p230 (Fig. 1A, Perturbation of transport by 3-MA) (Hirosako *et al.* 2004).

In practice, for the first round of screening, we screened kinase inhibitors to identify those that disrupted the localization of CI-M6PR (Fig. 1B, kinase inhibitor screening). A kinase inhibitor usually inhibits several types of kinase at its different concentrations. For example, Bisindolylmaleimide (BIM) at the concentration approximately 10 nM inhibits a protein kinase C (PKC) but it also inhibits protein kinase A (PKA) at high concentrations more than 2 μM. Taking the concentration-dependent broad specificity of kinase inhibitors into consideration, we carried out the kinase inhibitor screening at four different concentrations. The concentration for each kinase inhibitors were described in Table S1 in Supporting Information. In the second round of screening, we used the results of the first round to design RNAi experiments that would allow us to identify specific kinases or kinase regulators that were responsible for the phenotypes of interest (Fig. 1B, kinase siRNA screening).

To allow the imaging of as many samples as possible for large-scale screening applications and to acquire numerous imaging data in a systematic manner, we developed an automated high-throughput fluorescence-based cell imaging system using cell array chips. The system consisted of microchamber array chips, an automatic pipetting device (Fig. 1B, the CellTech Station, which was custom-made for us by Nikkyo Technos Co., Ltd), and an automatic system for the capture of fluorescent images. We used two types of imaging tool: an INCell Analyzer 1000 system (GE Healthcare Co., Ltd) and an LSM510 laser scanning confocal microscope (Carl Zeiss, Co., Ltd).

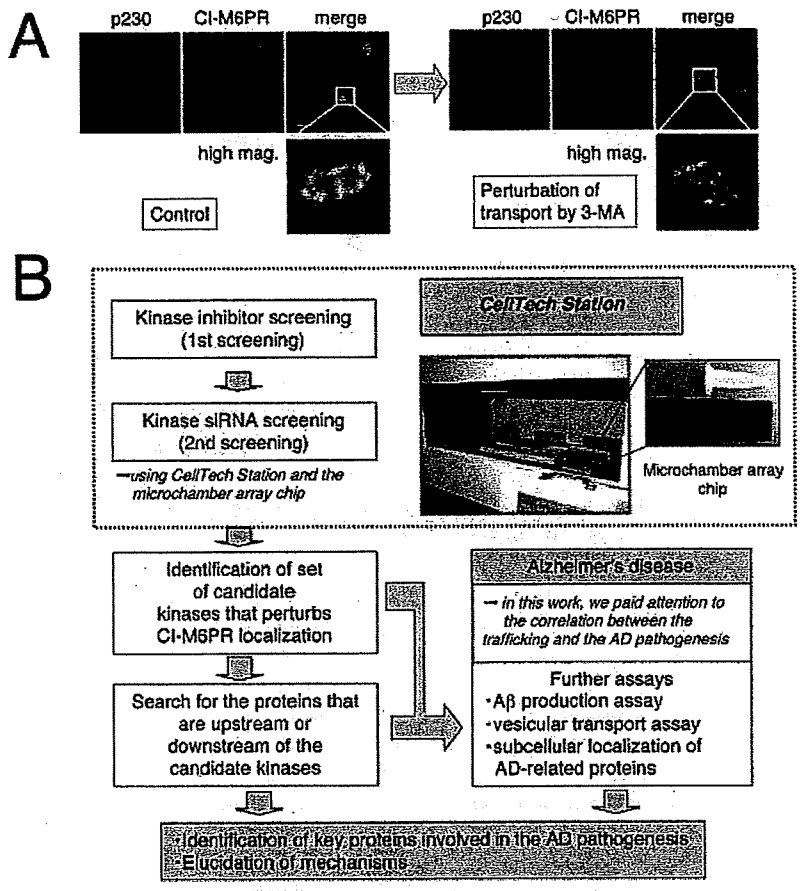


Figure 1 Flowchart for the identification of kinase-regulated pathways for the trafficking of CI-M6PR by high-throughput visual screening.

After identification of candidate kinases that perturbs CI-M6PR localization, we are going to investigate the relation between the defect in the trafficking of CI-M6PR and the AD pathogenesis using biochemical and cell biological studies (Fig. 1B, a panels on the bottom-right corner). Concurrently, we are going to search for the proteins that are upstream or downstream of the candidate kinases using available database or literature information network, and examine the role of the protein in the AD pathogenesis (Fig. 1B, a panel on the bottom-left corner). Considering all the protein information obtained above together, we are going to identify the key protein(s) involved in the AD pathogenesis.

Identification of kinase inhibitors that perturb CI-M6PR localization and TGN morphology

To determine the types of kinase that affect the intracellular localization of CI-M6PR or TGN morphology, we examined the effect of 29 different kinase inhibitors (Table S1 in Supporting Information) on the localization of CI-M6PR using our cell array chip-based automatic screening system. HeLa cells were first incubated with various kinase inhibitors in microchambers, and then fixed and immunostained with antibodies against p230 and CI-M6PR. Images were acquired using the INCell Analyzer 1000 or LSM510 confocal microscope, and the co-localization of CI-M6PR fluorescence (red) and p230 fluorescence (green) was analyzed by means of the co-localization index that is described in Fig. 2.

We identified the following five kinase inhibitors that disrupt the localization of CI-M6PR: Bisindolylmaleimide I (BIM) (Lee *et al.* 2005), SH-5 (Kierbel *et al.* 2005), staurosporine (Dangi & Shapiro 2005), GSK3β kinase inhibitor VII (Conde *et al.* 2003), and *4,5,6,7*-Tetrabromobenzotriazole (TBB) (Schermer *et al.* 2005). When HeLa cells were treated with these kinase inhibitors, with the exception of GSK3β kinase inhibitor VII, CI-M6PR became localized to punctate cytoplasmic structures, which are probably endosomal compartments, rather than

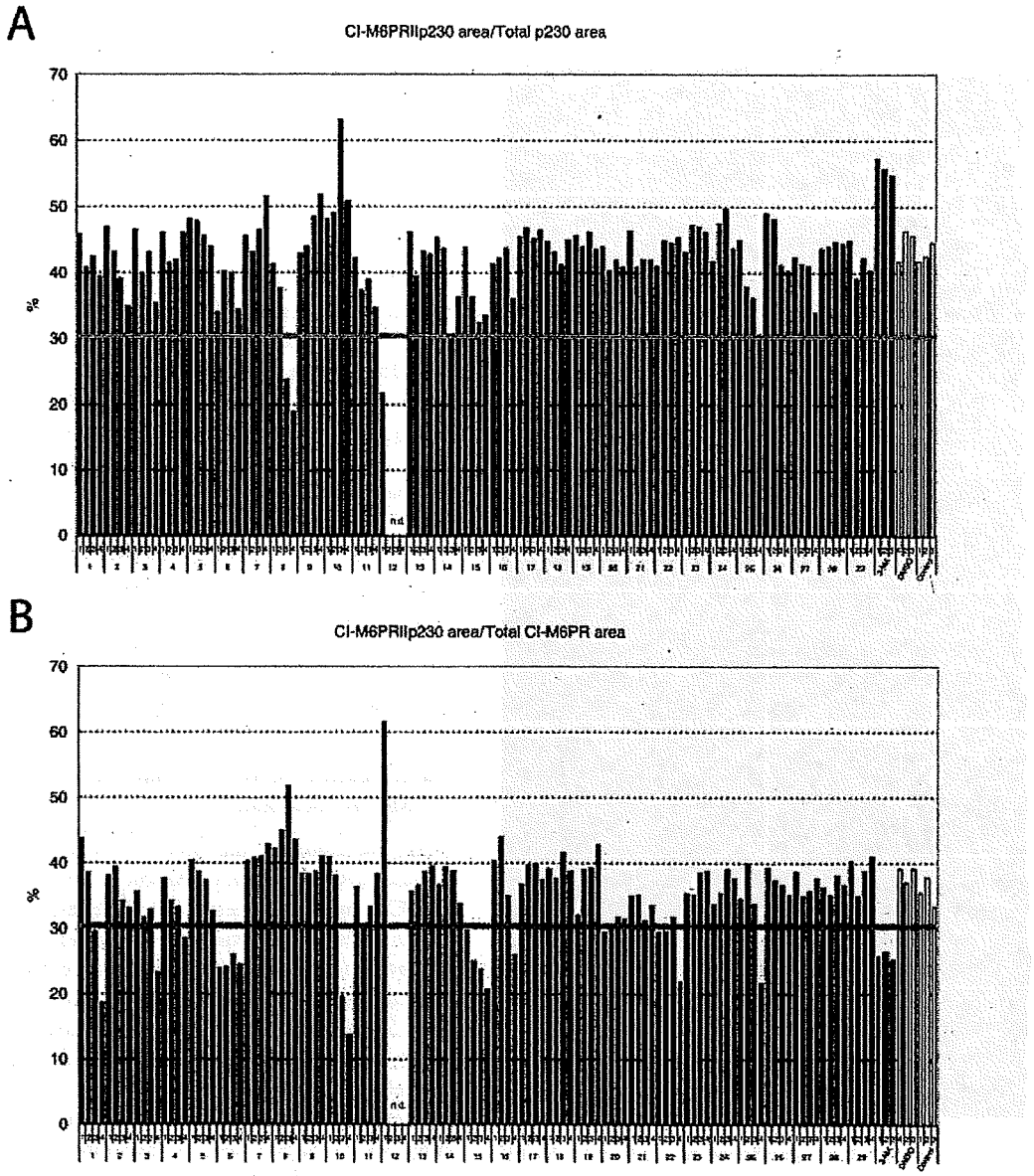


Figure 2 Co-localization index used in our visual screening to identify kinase inhibitors that perturb CI-M6PR localization in HeLa cells. (A) HeLa cells were treated with the kinase inhibitors that are listed in Table S1 in Supporting Information. We carried out immunostaining using antibodies against CI-M6PR and p230, and calculated the area in which CI-M6PR co-localized with p230 using the InCell Analyzer. The graph shows the percentage of the p230-positive area in which CI-M6PR is detected. The kinase inhibitors that gave a value of < 30% are Roscovitine (8) and JNK inhibitor II (12). (B) HeLa cells were treated with the kinase inhibitors at three different concentrations described in Table S1 in Supporting Information, and immunostained as described above. The graph shows the percentage of the CI-M6PR-positive area in which p230 is detected. Kinase inhibitors that gave a value of < 30% are as follows: H-89 (1), BIM (3), SH-5 (4), STS (6), Genistein (10), GSK3β VII (11), TBB (16), JNK inhibitor II (12), AG1296 (15), Calphostin C (22), and Sphingosine kinase inhibitor (25). We analyzed the fluorescent images of cells that had been treated with each kinase inhibitor, and found that the color of the inhibitors affected the measurements for 8, 12, and 22. In addition, the p230-positive Golgi apparatus was disrupted in 1, 10, 15, and 25. Taken together, we identified five kinase inhibitors that affect the localization of CI-M6PR, but not p230: BIM (3), SH-5 (4), STS (6), GSK3 β inhibitor VII (11), TBB (16). Red bar = 3-MA as a positive control; Yellow bar = DMSO; White bar = a negative control.

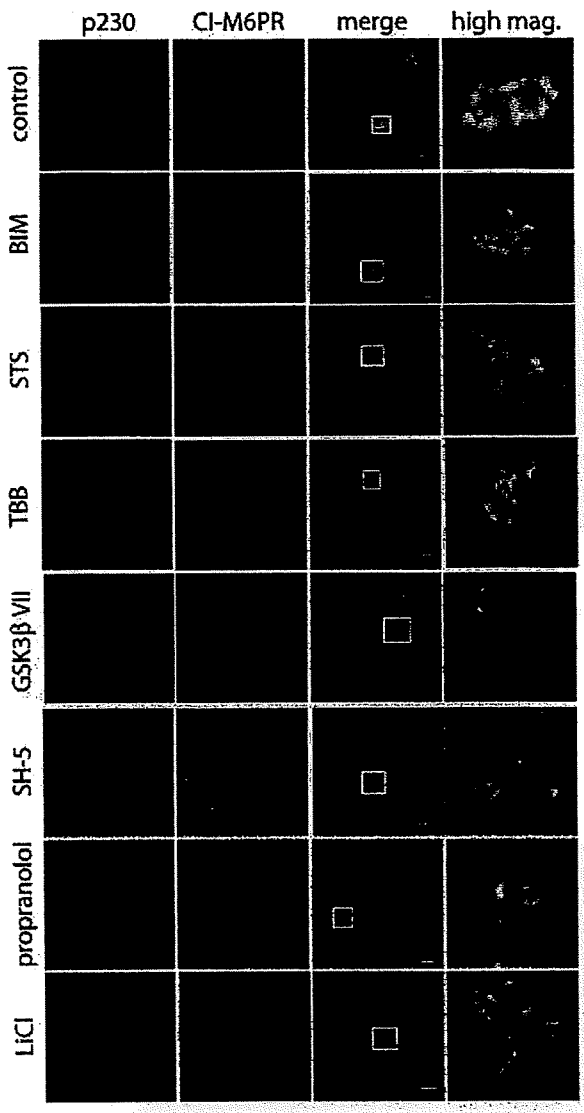


Figure 3 Validation of kinase inhibitors that perturb CI-M6PR localization in HeLa cells. HeLa cells that had been grown in microchambers were incubated in the presence of DMSO (14 mM, control) for 3 h, BIM (20 μM, BIM) for 0.5 h, staurosporine (2 μM, STS) for 2 h, TBB (200 nM, TBB) for 3 h, GSK-3β kinase inhibitor VII (50 μM, GSK3β VII) for 0.5 h, or SH-5 (10 μM, SH-5) for 2 h. HeLa cells that had been cultured in glass-based dishes were incubated with propranolol (100 μM, propranolol) for 1 h, or LiCl (20 mM, LiCl) for 3 h. After incubation, the cells in either the microchambers or the glass-based dishes were fixed and incubated with anti-p230 and anti-CI-M6PR antibodies, followed by Cy2- or Cy3-conjugated secondary antibodies, respectively. The samples were viewed using an LSM510 confocal microscope with a 63x Plan-Neofluar oil immersion objective.

to the TGN (Fig. 3). However, the localization of p230 did not change. BIM is a PKC inhibitor that also inhibits protein kinase A (PKA) at high concentrations (≥ 2 μM). Propranolol, which is another PKA inhibitor, resulted in the localization of CI-M6PR in punctate cytoplasmic structures rather than in the TGN (Fig. 3, Propranolol) (Saucerman *et al.* 2006). Staurosporine is a broad spectrum inhibitor of protein kinases. The cells appeared to become more rounded after treatment with staurosporine, but the localization of CI-M6PR changed in a similar manner to that seen with the other inhibitors. TBB is a specific casein kinase II (CK2) inhibitor. The retrieval of CI-M6PR from the endosomes to the TGN was reported to be controlled by the phosphorylation of phosphofurin acidic cluster sorting protein 1 (PACS-1) by CK2 (Scott *et al.* 2006). GSK3β kinase inhibitor VII is a specific inhibitor of GSK3β kinase, and caused fragmentation of the TGN and the apparent partial segregation of the two markers (Fig. 3; GSK3β kinase inhibitor VII). The same phenotype was observed after treatment with LiCl, another GSK3β inhibitor (Fig. 3; LiCl) (Cheng *et al.* 2008). As a result, we chose three types of kinase for the subsequent RNAi experiments: protein kinases of the AGC group, CK2, and GSK3β.

Identification of kinases that are involved in CI-M6PR localization

Next, we examined the effect of siRNAs (*Silencer* Human Kinase siRNA Library; Ambion) for 80 kinase genes (AGC group, 76 genes; CK2, 3 genes; GSK3β, 1 gene; the names of the kinases are given in Table S2 in Supporting Information) on CI-M6PR localization/sorting using our automatic visual screening system with the LSM510 confocal microscope as the imaging system. With our RNAi system, the efficiency of gene silencing in HeLa cells was found to be more than 90%, as measured morphologically by the knockdown of kinesin family member 11 (KIF11) (Fig. S1 in Supporting Information). We identified five kinases whose knockdown perturbed the localization of CI-M6PR: CDC42 binding protein kinase-β (CDC42BPB), protein kinase, cAMP-dependent, catalytic-α (PRKACA), protein kinase, cAMP-dependent, catalytic-γ (PRKACG), GSK3β, and CSNK2A1 (casein kinase 2, alpha 1 polypeptide).

CDC42BPB regulates cell polarity by controlling the dynamics of actin and myosin (Gomes *et al.* 2005; Wilkinson *et al.* 2005). PRKACA and PRKACG are catalytic subunits of PKA, which regulates the dynamics of many organelles through its interaction with A-kinase anchoring protein (Wong & Scott 2004). GSK3β regulates microtubule dynamics and cellular responses, including

protein synthesis, gene expression, and protein degradation (Akhmanova *et al.* 2001; Fumoto *et al.* 2006). CSNK2A1 is one of the subunits of CK2, which was reported to regulate CI-M6PR trafficking in HeLa cells (Scott *et al.* 2006). We confirmed that the protein levels of these five kinases were reduced in the cells that had been transfected with the appropriate siRNAs compared to those in cells that had been transfected with an siRNA against enhanced green fluorescent protein (eGFP) (Fig. S2A in Supporting Information).

Gene silencing of CDC42BPB induced the redistribution of CI-M6PR from the TGN to cytoplasmic structures (Fig. 4, CDC42BPB), but also resulted in the partial disruption of the Golgi apparatus. Gene silencing of PRKACA, PRKACG or CSNK2A1 changed the localization of CI-M6PR from the TGN to cytoplasmic structures (Fig. 4, PRKACA, PRKACG, CSNK2A1). GSK3β knockdown caused the fragmentation of the TGN that contained both CI-M6PR and p230 (Fig. 4, GSK3B).

We examined the co-localization of CI-M6PR with various organelle markers in kinase-knockdown HeLa cells by immunofluorescence. The following organelle markers were used: EEA1 (early endosome antigen 1) as an early endosome marker, 2,2′-dioleoyl lysobisphosphatidic acid (LBPA) as a late endosome marker, and Lamp2 as a lysosomal marker. In control HeLa cells, none of the organelle markers (EEA1, LBPA and Lamp2) overlapped with the CI-M6PR-positive punctate structures (Fig. S3, B and C; control in Supporting Information). In these cells, CI-M6PR localized primarily to the TGN and to specialized vesicles, but not to the early/late endosomes as had been shown in another report (Hirosako *et al.* 2004). In the knockdown cells, CI-M6PR co-localized partially with EEA1 in the peripheral cytoplasmic vesicles, but not with the other markers (Fig. S3A–C in Supporting Information). The change in localization of CI-M6PR in the GSK3β-knockdown cells was of particular interest. In these cells, the majority of the fluorescent signal for all the marker proteins appeared to be clustered in the central region of the cells, near the centrosome. CI-M6PR co-localized partially with EEA1 and LBPA in this central region in the knockdown cells (Fig. S3A–C; GSK3B in Supporting Information).

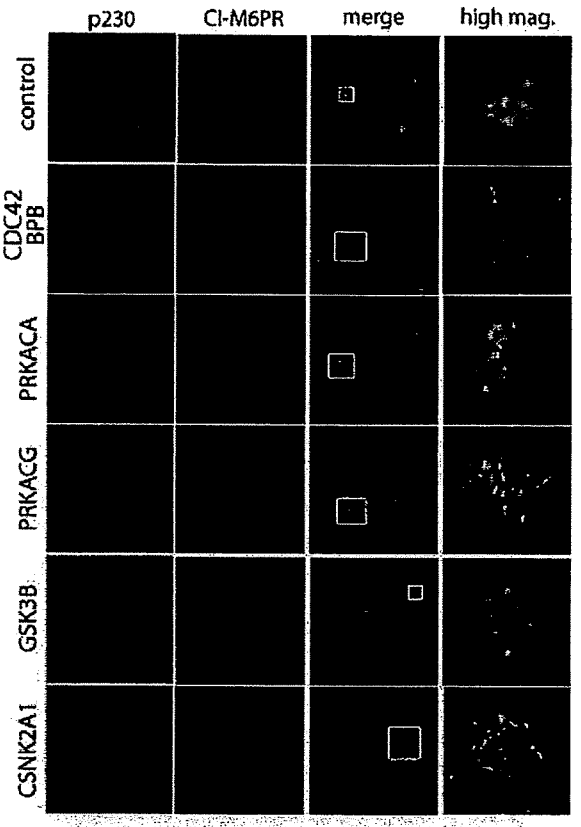


Figure 4 Visual screening to identify kinase siRNAs that perturb CI-M6PR localization in HeLa cells. HeLa cells that had been grown in microchambers were transfected with 50 nM siRNA against a control protein (eGFP), or against CDC42BPB, PRKACA, PRKACG, GSK3β, or CSNK2A1. After incubation for 72 h in culture medium, the cells were fixed and incubated with anti-p230 and anti-CI-M6PR antibodies, which were visualized by Cy2- and Cy3-conjugated secondary antibodies, respectively. The samples were viewed using an LSM510 confocal microscope with a 63x Plan-Neofluar oil immersion objective. Bar = 10 μm.

Effects of gene silencing of the identified kinases on APP processing

We next investigated the relation between the perturbations in the trafficking of CI-M6PR that were induced by kinase knockdown and the pathogenic processing of Aβ using HEK-APP cells, which stably express Swedish mutant APP (APPsw). We confirmed that the protein level of each kinase was decreased in the kinase-knockdown cells (Fig. S2B in Supporting Information).

Initially, to detect the final products of APP processing, we measured the amount of Aβ40 and Aβ42 that had been secreted into the medium using a sandwich ELISA. Gene silencing of PRKACG and GSK3β increased the amounts of secreted Aβ40 and Aβ42 by approximately twofold in both cases, compared with that secreted by the control cells (Fig. 5A). Gene silencing of the other kinases had no effect on the amount of Aβ40 or Aβ42 that was secreted.

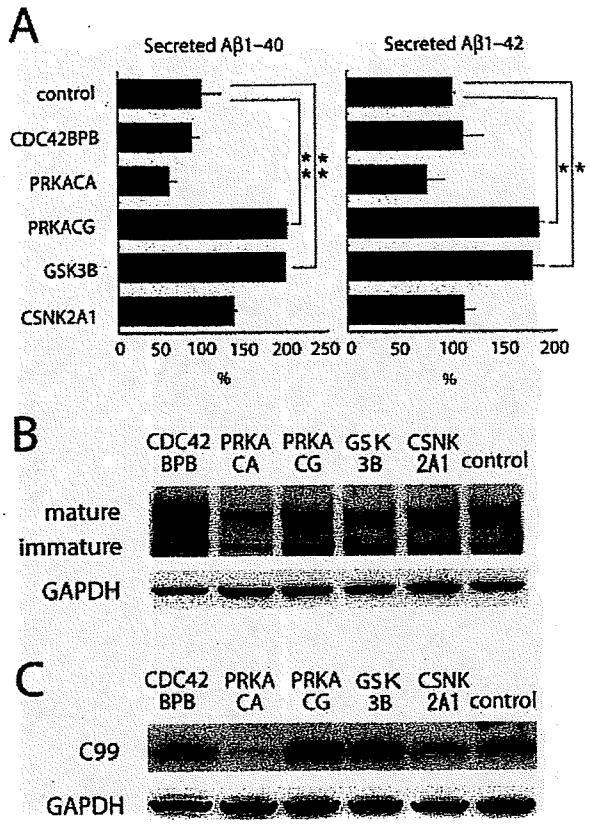


Figure 5 Effects of kinase knockdown on the secretion of Aβ40 or Aβ42 into the medium (A), APP maturation (B), or β-secretase activity (C). (A) HEK-APP cells were plated on poly-L-lysine-coated 6-well plates and transfected with 50 nM siRNA for each kinase indicated. After incubation for 72 h, the medium was replaced with fresh medium. Six hours after the medium was exchanged, the medium and cells were collected. The levels of secreted Aβ40 and Aβ42 in the medium were measured by ELISA and normalized against the concentration of protein in the cell lysate. Values are means ± S.E.; $n = 3$, $*P < 0.05$, $**P < 0.005$, *t*-test. (B) After incubation for 72 h, the cells were lysed and the cell proteins immunoblotted using a mouse monoclonal antibody (AB5352) against the C-terminus of APP to detect APP maturation. (C) After incubation for 72 h, the cells were lysed and cell proteins immunoblotted using a monoclonal antibody (82E1) against the N-terminus of human Aβ to detect the C99 fragment. GAPDH was used as a loading control for both experiments.

Next, we examined the maturation of APP in HEK-APP cells by Western blotting. With the lysate from the control cells, both the mature form and the immature form of APP were detected on the western blot. The immature form of APP is predominantly *N*-glycosylated and localized in the endoplasmic reticulum (ER) and the *cis*-Golgi. The mature form is an *N*,O-glycosylated species that is concentrated in the TGN/PM (Weidemann *et al.* 1989). Gene silencing of CDC42BPB or PRKACG resulted in an increase in the level of the mature form (Fig. 5B). In contrast, the knockdown of PRKACA caused a reduction in the total amount of APP protein. Gene silencing of GSK3β or CSNK2A1 had no effect on the maturation compared with that in control cells (Fig. 5B, GSK3B and CSNK2A1).

Finally, we examined β-secretase activity in the cells. The activity was estimated by measuring the amount of the APP C-terminal C99 fragment, which is produced by β-secretase, in the cells. The C99 fragment was detected by Western blotting with the 82E1 monoclonal antibody, which reacts with the C99 fragment but does not react with the APP C-terminal fragment that is produced by α-secretase. We found that gene silencing of PRKACG or GSK3β increased the level of the C99 fragment compared with that in control cells. Taken together with the results of the APP processing experiments, we concluded that the knockdown of PRKACG or GSK3β induced a substantial increase in Aβ secretion. It remains possible that the other kinases (CDC42BPB, PRKACA and CSNK2A1) that have been implicated in CI-M6PR trafficking could be involved in the pathogenesis of CI-M6PR-related diseases other than AD.

Next we examined the subcellular localization of the AD-related protein, BACE1, in PRKACG- and GSK3β-knockdown cells because there had been many reports that suggested a positive correlation between the perturbation of membrane trafficking of BACE1 and the pathogenesis of AD, and because CI-M6PR and BACE1 contain the same sorting signal in their cytoplasmic region (Shiba *et al.* 2004; He *et al.* 2005; Tesco *et al.* 2007). To monitor the localization of BACE1, we expressed myc-tagged BACE1 protein in HeLa cells, and carried out indirect immunofluorescence analysis using an anti-myc antibody. In control cells, BACE1 localized mainly to the juxtanuclear region and partially to the PM. We found by dual-immunofluorescence analysis that BACE1 co-localized with EEA1 in the juxtanuclear region, but not in the cytoplasmic vesicles (Fig. 6, control). In contrast, in PRKACG-knockdown cells, the majority of the BACE1 was localized not in the juxtanuclear region but in cytoplasmic vesicles, where it partially co-localized with EEA1 (Fig. 6, PRKACG). In the GSK3β-knockdown cells, almost all the fluorescent signal from BACE1 co-localized with that from EEA1, and the BACE1/EEA1-positive vesicles appeared to be clustered in the central region of the cells, near the centrosome (Fig. 6, GSK3B), as was the signal from CI-M6PR.

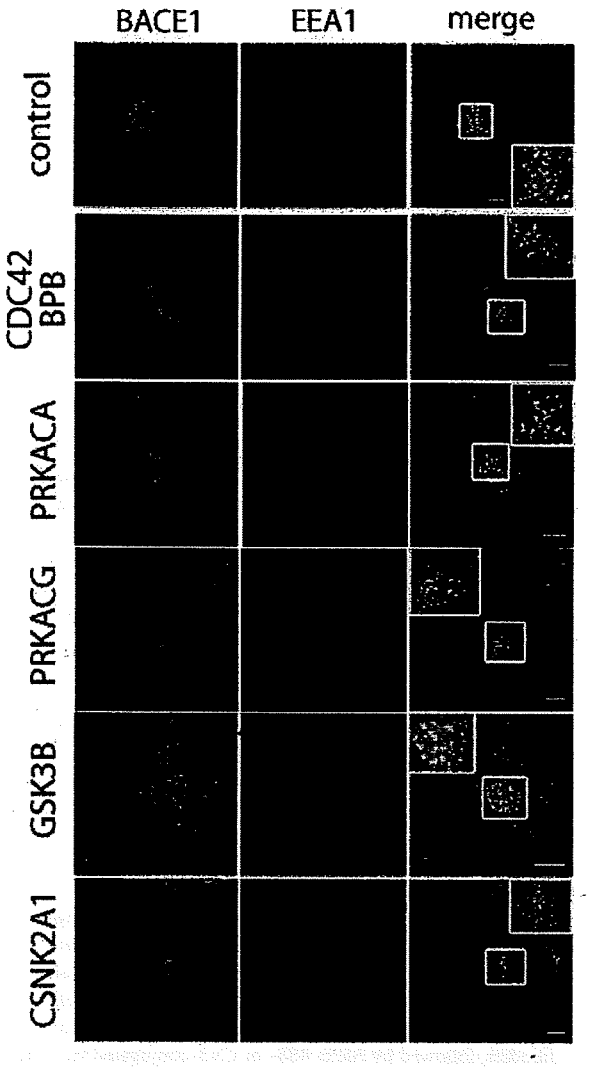


Figure 6 Changes in the subcellular localization of BACE1 and EEA1 in kinase-knockdown HeLa cells. HeLa cells that had been grown on glass-based dishes were transfected with 50 nM siRNA against a control protein (eGFP), PRKACG, or GSK3β. The cells were further transfected with 1 μg/mL BACE1-myc plasmid at 48 h after transfection with the siRNAs. After further incubation for 24 h, the cells were fixed and dual-immunostained with anti-myc and anti-EEA1 antibodies, followed by Alexa 488- or Cy3-conjugated secondary antibodies, respectively. The samples were viewed using an LSM510 confocal microscope with a 63x Plan-Neofluar oil immersion objective. Scale bar = 10 μm.

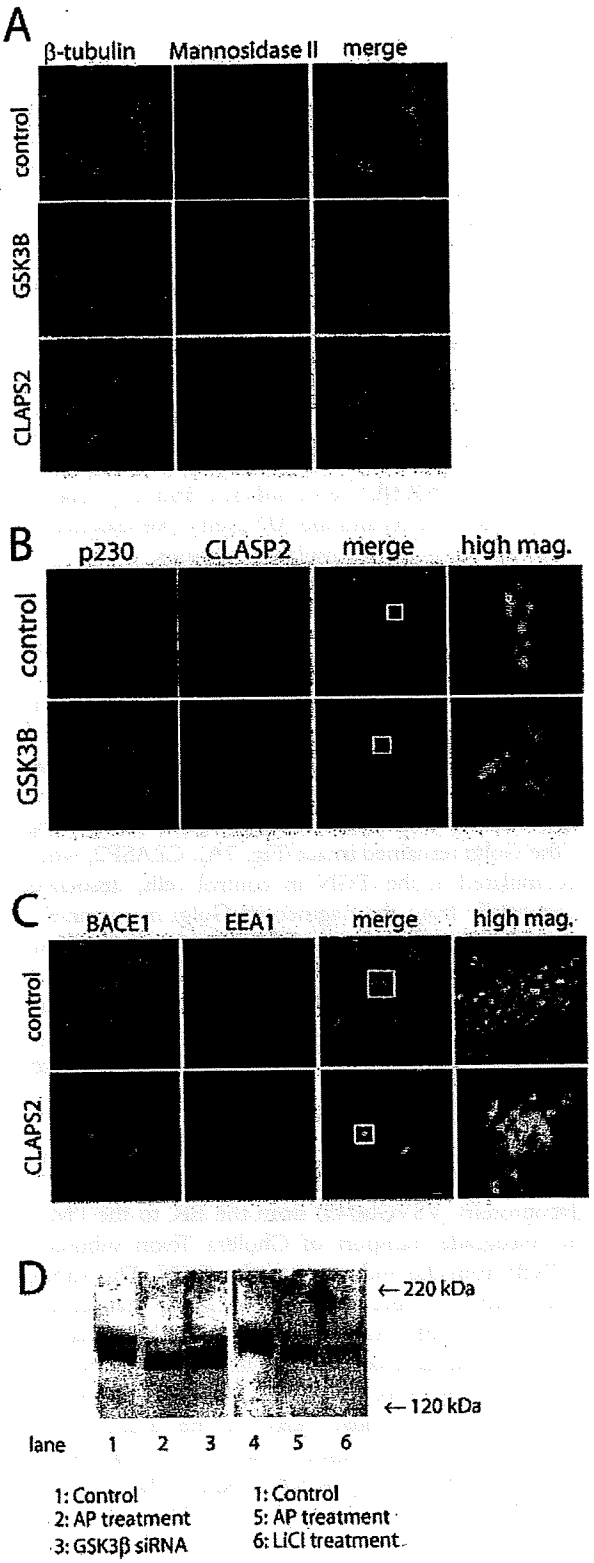
Functional modulation of CLASP2 by GSK3β is involved in Aβ secretion

To investigate the relation between GSK3β or PRKACG knockdown and increased Aβ secretion, we searched for proteins whose function is regulated by the kinases and that are involved in the vesicular transport between endosomes and the TGN.

As a result, we focused on the function of CLASP2, which is a microtubule binding protein (Efimov *et al.* 2007). CLASP2 contains several potential GSK3β phosphorylation motifs and its function could be regulated by GSK3β. CLASP2 associates specifically with the TGN protein GCC185 and functions in the formation of the asymmetric microtubule network in polarized cells. GCC185 was found to play a crucial role both in the retrograde transport of Shiga toxin and CI-M6PR between the recycling endosomes and the TGN, and in the maintenance of Golgi morphology (Akhmanova *et al.* 2001; Mimori-Kiyosue *et al.* 2005; Wittmann & Waterman-Storer 2005; Reddy *et al.* 2006; Derby *et al.* 2007). In contrast to GSK3β, we could not find any relation between PRKACG and the AD even if we searched for a relationship using the available databases.

First we examined the relation between GSK3β activity and CLASP2 function. We found that GSK3β knockdown reduced the number of centrosomally focused microtubules (oriented microtubule networks around the centrosome) and simultaneously induced a slight fragmentation of the Golgi apparatus (Fig. 7A) as reported previously (Fumoto *et al.* 2006). We also found that CLASP2-knockdown cells showed the same morphological phenotypes with regard to the centrosomal microtubules but the morphology of the Golgi remained intact (Fig. 7A). CLASP2, which accumulated at the TGN in control cells, dissociated substantially from the fragmented Golgi membranes in GSK3β-knockdown cells (Fig. 7B). In addition, we detected a substantial inhibitory effect on CLASP2 phosphorylation in GSK3β-knockdown or lithium chloride-treated cells (Fig. 7D). These results suggest that a decrease in GSK3β activity could modulate the localization of CLASP2 to the TGN and its function.

Next we examined the effects of the knockdown of CLASP2 on either the anterograde transport of a temperature sensitive version of the vesicular stomatitis virus glycoprotein (VSVGts045) from the ER to the PM or the retrograde transport of Cholera Toxin subunit B (CTxB) from the endosome to the TGN. The knockdown had no effect on the anterograde transport of VSVGts045-GFP (Adachi *et al.*, unpublished data) but caused substantial retardation of the retrograde transport of CTxB (Fig. S4B, siRNA CLASP2 in Supporting Information). A similar delay in the transport was observed in GSK3β-knockdown cells (Fig. S4A in Supporting Information, siRNA GSK3B). The localization of BACE1 in the CLASP2-knockdown cells was perturbed in a similar manner as in the GSK3β-knockdown cells



(Fig. 7C). Finally, we measured the secretion of Aβ40 and Aβ42 into the medium. CLASP2 knockdown increased the amounts of secreted Aβ40 and Aβ42 by approximately 2.0-fold and 1.4-fold, respectively, as compared with the control (Fig. 8A). These results suggested that a decrease in GSK3β activity might modulate the activity of CLASP2 in microtubule dynamics or in the TGN, which would in turn result in the mislocalization of BACE1 and an extensive secretion of Aβ.

We compared the coalescence of APP and BACE1 in GSK3β- or CLASP2-knockdown cells to that in control cells morphologically using an immunofluorescence

Figure 7 Effects of gene silencing of GSK3β or CLASP2 on the morphology of microtubules and the Golgi apparatus in HeLa cells (A), on the dissociation of CLASP2 from the Golgi membranes (B), on the changes in the subcellular localization of BACE1 and EEA1 (C), or on the phosphorylation states of CLASP2 (D). (A) GSK3β- or CLASP2-knockdown HeLa cells were fixed and dual-immunostained with antibodies against anti-β-tubulin and anti-mannosidase II (medial Golgi marker), followed by Alexa 488- or Cy3-conjugated secondary antibodies, respectively. The samples were viewed using an LSM510 confocal microscope with a 63x Plan-Neofluar oil immersion objective. Scale bar = 10 μm. GSK3β or CLASP2 knockdown reduced centrosomally-focused microtubules (oriented microtubule networks around the centrosome). GSK3β knockdown caused the disassembly of the Golgi apparatus, but CLASP2 knockdown did not. (B) GSK3β knockdown induced a slight fragmentation of the Golgi apparatus, which occurred concomitantly with the dissociation of CLASP2 from the TGN membranes. (C) HeLa cells that had been grown on glass-based dishes were transfected with 50 nM siRNA against a control protein (eGFP) or CLASP2. The cells were further transfected with 1 μg/mL BACE1-myc plasmid at 48 h after transfection with the siRNAs. After further incubation for 24 h, the cells were fixed and dual-immunostained with anti-EEA1 and anti-myc antibodies, followed by Alexa 488- or Cy3-conjugated secondary antibodies, respectively. The samples were viewed using an LSM510 confocal microscope with a 63x Plan-Neofluar oil immersion objective. Scale bar = 10 μm. The localization of BACE1 in CLASP2-knockdown cells was perturbed as had been observed in the GSK3β-knockdown cells (compare with the image in Supporting Information Fig. S6, GSK3B). (D) The inhibition of phosphorylation of CLASP2 in the GSK3β-knockdown HeLa cells was detected by the treatment of cell lysates with alkaline phosphatase (AP). We examined the effect of decreasing the activity of GSK3β by siRNA or lithium chloride on two protein bands. As shown in the figure, two CLASP2-positive bands were observed in HeLa cell lysate (lane 1). Interestingly, the upper band disappeared and shifted to the lower position upon AP treatment (lane 2). The same shift was observed in the lysate of GSK3β-knockdown cells (lane 3). Treatment with lithium chloride also induced the same shift of bands (lane 4, control; lane 5, AP treatment; lane 6, LiCl treatment). Therefore, the upper band of CLASP2 in the blot might correspond to phosphorylated CLASP2.

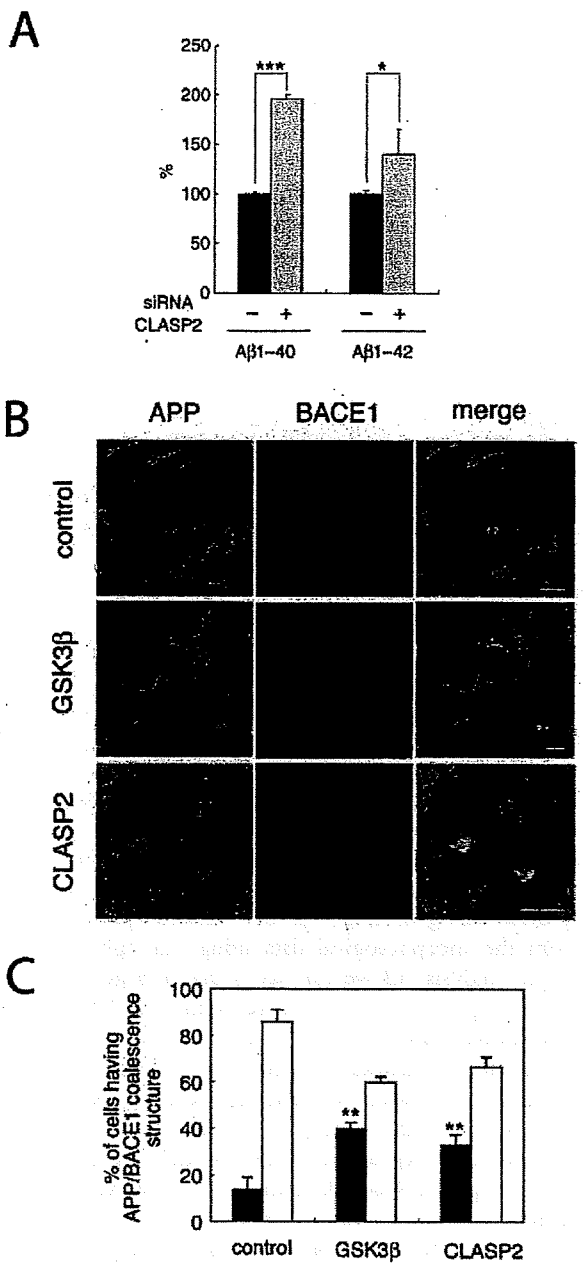


Figure 8 Effects of CLASP2 knockdown on Aβ40 or Aβ42 secretion into the medium (A), or the coalescence of BACE1 and APP in endosomes (B, C). (A) HEK-APP cells were plated on poly-L-lysine-coated 6-well plates and transfected with 50 nM siRNA against CLASP2. After incubation for 72 h, the medium was replaced with fresh medium. Six hours after the medium was exchanged, the medium and cells were collected. The levels of secreted Aβ40 and Aβ42 in the medium were measured by ELISA and normalized against the concentration of protein in the cell lysate. Values are means ± SE; $n = 3$, $*P < 0.05$, $**P < 0.005$, *t*-test. (B) HEK-APP cells that had been grown on glass-based dishes were transfected with 50 nM siRNA against either a control protein (eGFP) or CLASP2. The cells were further transfected with 1 μg/mL BACE1-myc plasmid at 48 h after the initial siRNA transfection. After further incubation for 24 h, the cells were fixed and dual-immunostained with anti-APP and anti-myc antibodies, which were visualized by Alexa 488- or Cy3-conjugated secondary antibodies, respectively. The samples were viewed using an LSM510 confocal microscope with a 63× Plan-Neofluar oil immersion objective. Scale bar = 10 μm. The knockdown of CLASP2 induced the extensive coalescence of APP and BACE1 in the centrosomal region of the cells. (C) The percentage of the cells in which the co-localization of APP and BACE1 was observed was compared with that of the control cells using morphometric analysis. BACE1 in control cells did not co-localize with APP. In contrast, extensive co-localization of these proteins was observed in GSK3β- or CLASP2-knockdown cells. Values are means ± SE; $n = 3$, $*P < 0.05$, $**P < 0.005$, *t*-test.

method. In control cells, BACE1 did not co-localize with APP, rather the fluorescence signal of BACE1 appeared to be segregated completely. In contrast, substantial, but not complete, co-localization of the two fluorescence signals was observed in GSK3β- or CLASP2-knockdown cells (Fig. 8B,C).

Discussion

In this article, we describe the development of a versatile functional screening system to examine the vesicular trafficking of CI-M6PR and its regulating kinase by detecting changes in the localization of the protein. As a result, we identified two kinase-regulated trafficking pathways that were involved in the pathogenesis of AD through the activation of Aβ secretion; a PRKACG-regulated pathway that was associated with aberrant accumulation of matured APP in the cells and a GSK3β-regulated pathway that was associated with the accumulation of BACE1-containing endosomes near the juxtanuclear region.

PKA has been implicated in the pathological hyperphosphorylation of tau on Ser214 and 409, which leads to the formation of neurofibrillary tangles in AD (Wang *et al.* 2007). In addition, Su *et al.* (2003) showed that H-89 (a PKA inhibitor) inhibited the maturation of APP, which involves O-glycosylation during a post-Golgi stage of the secretory pathway, and secretion of Aβ. However, the role of PKA during APP processing is not clear. In this study, we demonstrated the silencing of PRKACG, which is a catalytic subunit of PKA, increased the mature form of APP (Fig. 5B) and activated Aβ secretion (Fig. 5C).

GSK3β has been shown to play a crucial role in the phosphorylation of tau and the modulation of APP

processing (Phiel *et al.* 2003; Noble *et al.* 2005; Rockenstein *et al.* 2007; Wang *et al.* 2007), which suggests that abnormal levels and activity of GSK3β may be associated with the pathology of AD. However, there have been very few reports that have examined the relation between GSK3β activity and a specific trafficking pathway for AD-related proteins. This is the first time that a positive relation between a GSK3β-regulated membrane trafficking pathway and Aβ secretion has been demonstrated.

We searched for proteins whose function is regulated by PRKACG or GSK3β and that are involved in the vesicular transport between endosomes and the TGN. We could not find any association between PRKACG and AD even if we searched for a relationship using the available databases. However, we found that a functional defect in CLASP2, which arose due to the modulation of GSK3β activity, might perturb membrane trafficking between the endosomes and the TGN through its deficient interaction with the Golgi associating protein GCC185. Therefore, we attempted to gain further mechanistic insight at a molecular level into how GSK3β and CLASP2 control the specific trafficking pathway that is involved in the pathogenic secretion of Aβ. As a result, we found that the modulation of GSK3β-mediated phosphorylation of CLASP2 resulted in the mislocalization of BACE1 and extensive Aβ secretion (Fig. 8). There has been reported that the modulation of GSK3β-mediated phosphorylation of CLASP2 results in a significant increase in CLASP2 signal at distal microtubule ends (plus ends) and is involved in the local regulation of microtubule dynamics (Akhmanova *et al.* 2001; Wittmann & Waterman-Storer 2005). In addition, we observed that GSK3β-knockdown reduced the number of centrosomally-focused microtubules and induced the sight fragmentation of the Golgi apparatus (Fig. 7A). These results indicate that the modulation of GSK3β-mediated phosphorylation of CLASP2 might cause the defect in the microtubule-dependent membrane trafficking pathways between the Golgi and endosomes. In fact, we observed substantial retardation of the retrograde transport of CTxB from endosomes to the TGN (Fig. S4B in Supporting Information). The defect in the transport pathways might cause an extensive coalescence of APP and BACE1 in the juxtanuclear region of GSK3β- or CLASP2-knockdown cells and result in an extensive Aβ secretion. At this time, we cannot elucidate the causal correlation between the defect in the trafficking pathways and the promotion of coalescence of APP and BACE1. More biochemical studies are needed to solve the issue.

There have been several reports that demonstrated that the inhibition of clathrin-mediated endocytosis reduces β-cleavage of APP and thus Aβ production (Ehehalt *et al.* 2003; Rajendran *et al.* 2006). It was suggested that APP and BACE1 are combined together after endocytosis by the clustering and coalescence of APP- and BACE1-containing rafts within endosomes (Hattori *et al.* 2006). A significant fraction of BACE1 is known to localize to lipid rafts and amyloidogenic processing of APP might occur within these rafts. As shown in Fig. 8(B,C), our immunofluorescence analysis showed that a large extent of fluorescence signal of APP appeared to coalesce with that of BACE1 in GSK3β- or CLASP2-knockdown cells. At this time, we could not clarify whether both APP and BACE1 are in the rafts of "same membrane vesicles". Hattori *et al.* (2006) reported that BACE1 and APP cluster and coalesce each other in the lipid raft at endosomes. As shown in Fig. S3A in Supporting Information, we observed that both BACE1 and APP were in the EEA1-positive membranous structures, suggesting the possibility that the membranous structures, which gathered at the juxtanuclear region, are lipid rafts at early endosomes. One possible experiment is to perform the co-fractionation experiment using sucrose density gradient. However, we believe that it would be very difficult to confirm the precise coalescence of APPs with BACE1 in the rafts of "same membrane vesicles". Even if BACE1 and APP were detected at the same fraction, we could not clarify whether these proteins exist at the same membrane vesicles or not. To solve this issue, we need to isolate the membranous structures in the juxtanuclear region and/or to confirm morphologically the co-localization of BACE1/APP in the lipid raft using immuno-electron microscopic analysis. From the morphological data using our light microscopic analysis, all we can say is that a large amount of APP appears to co-localize with BACE1 in the knockdown cells.

Recently, Fernández-Medarde *et al.* (2007) have shown a possible correlation between the decreased expression of CLASP2 and the pathogenesis of AD. They compared the levels of expression of individual genes in the hippocampus of Ras-GRF1 knockout mice, which have defects in memory consolidation, with those in the hippocampus of control mice by using microarray analysis. In this study, the Clasp2 gene was found to be heavily down-regulated and, taken together with the proposed functions of other genes that were found to be expressed differentially in these mice, CLASP2 protein is involved in neurodegenerative diseases such as AD. However, the authors supposed that the decreased expression of CLASP2 might contribute to microtubule destabilization and result in the formation of neurofibrillary tangles by phosphorylating tau during AD pathogenesis. There are few publications to date that demonstrate a positive relationship

among defect in membrane trafficking, decreased activity of GSK3β, and excessive Aβ secretion. Our findings will shed light on the effect of the defect in membrane trafficking on AD pathogenesis.

We found that CDC42BPB, PRKACA, and CSNK2A1 did not enhance Aβ secretion even though they all perturbed the retrograde transport of CI-M6PR between the endosomes and the TGN. This suggests that a defect in the trafficking pathway is not sufficient for the increased secretion of Aβ. The trafficking pathways that are affected in CDC42BPB-, PRKACA-, or CSNK2A1-knockdown cells may be involved in the pathogenesis of diseases other than AD. Further studies are necessary to connect the kinases with their related diseases.

Our novel screening and analytical system has several notable features. Firstly, we will be able to identify the essential pathogenetic proteins that are upstream or downstream of the kinase because the protein kinase network has been studied extensively and many useful databases are available for general use. Secondly, the visual screening and assay system that we have described in this article is applicable to various types of localizomics studies. Several systematic screening methods that are based on gene silencing techniques such as RNAi have currently been established. In particular, recent advances in high-throughput imaging techniques and methods for image analysis have allowed morphological changes or dynamic processes such as mitosis and endo/exocytosis to be characterized in great detail by fluorescence microscopy (Pelkmans *et al.* 2005; Balklava *et al.* 2007). Our developed CellTech Station can do cell culture, transfection of siRNAs and plasmids, and indirect immunofluorescence analysis automatically, with the computational programs. It is highly possible that the functions associated with the CellTech Station are suitable for the various localizomics studies. By analyzing the localization of pathogenetically-important proteins, lipids, or mRNAs after the transfection of siRNAs against kinases, phosphatases, or other types of protein using our system, it will be possible to clarify the molecular links that are between the localization of these proteins and other macromolecules and the pathogenic mechanisms of related diseases.

Experimental procedures

Automatic visual screening system using the CellTech Station

To acquire numerous imaging data in a systematic manner, we established an automated high-throughput fluorescence-based cell imaging system using cell array chips. The system consisted of microchamber array chips, automatic sample preparation equipment (the CellTech Station, which was custom-made for us by Nikkyo Technos Co., Ltd), and automatic fluorescence-image acquisition systems (Fig. 1B). Briefly, 48 microchambers (12 × 4) with a diameter of 2.5 mm and an inter-chamber distance of 3.0 mm were arrayed on a black-quartz chip (Fig. 1B). The bottom of each microchamber was made of transparent quartz (thickness, 0.15 mm), which allowed us to detect fluorescence images within the UV range using an oil immersion lens. The CellTech Station was used to do cell culture, transfection of siRNAs and plasmids, and indirect immunofluorescence analysis automatically, using different computational programs.

To acquire images of the cells in the microchambers, we used an INCell Analyzer 1000 system (GE Healthcare Co., Ltd) or LSM510 confocal microscopy system (Carl Zeiss Co., Ltd). The advantage of this system was the ability to obtain multi-colored images of the 48 microchambers at high speed using a ×40 objective lens. By using the automatic sample preparation system, we could minimize the occurrence of discrepancies between the microchambers that were due to operational mistakes caused by human error.

Chemicals, antibodies and plasmids

The kinase inhibitors H-89, 3-methyladenine, and propranolol were purchased from Sigma Aldrich, and other kinase inhibitors from Calbiochem. These inhibitors were diluted to appropriate concentrations in DMSO and stored at –20 °C.

The antibodies that were used for western blotting were as follows: mouse polyclonal anti-CDC42BPB antibody (Abnova), mouse polyclonal anti-PRKACA antibody (Abnova), mouse polyclonal anti-PRKACG antibody (Abnova), rabbit polyclonal anti-CSNK2A1 antibody (BL753; Bethyl Laboratories), rabbit polyclonal anti-GSK3β antibody (Cell Signaling Technology), rabbit polyclonal anti-APP antibody (AB5352; Chemicon), mouse monoclonal anti-human amyloid β antibody (82E1; IBL), mouse monoclonal anti-GAPDH antibody (MAB374; Chemicon). HRP-conjugated anti-mouse and anti-rabbit antibodies from Upstate Biotechnology and Millpore, respectively, were used as secondary antibodies.

The antibodies that were used for immunofluorescence were as follows: rabbit polyclonal anti-CI-M6PR antibody (IBL), mouse monoclonal anti-p230 antibody (BD Biosciences), mouse monoclonal anti-EEA1 antibody (BD Biosciences), mouse monoclonal anti-Lamp2 antibody (H4B4; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit polyclonal anti-myc antibody (Cell Signaling). The mouse monoclonal anti-LBPA antibody was a gift from Dr. Toshihide Kobayashi (RIKEN). The anti-CLASP2 polyclonal antibody was a gift from Dr. Irina Kaverina (Vanderbilt University Medical Center). Cy2- and Cy3-conjugated anti-mouse antibodies (Chemicon), a Cy3-conjugated anti-rabbit antibody (Chemicon), and an Alexa 488-conjugated anti-rabbit antibody (Invitrogen) were used as secondary antibodies.

The VSVGts045-GFP construct was a gift from Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD).

Cell culture and transfection

HEK293 cells that stably expressed APPsw, and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Nissui) supplemented with 10% fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO). Geneticin (1 mg/mL; Invitrogen) was also required for the HEK293 stable transfectant. For plasmid transfection into the kinase-knockdown cells, cells that had been incubated for 48 h after transfection with the siRNAs were transfected with 1 μg/mL plasmid using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions.

RNAi manipulations

For siRNA screening, knockdown of the kinases by RNAi was carried out by the reverse transfection method using siPORT *NeoFX* Transfection Agent (Ambion) and 30 nM siRNA (*Silencer*® Human Kinase siRNA Library V3; Ambion) according to the manufacturer's instructions. Briefly, a mixture of Transfection Agent and siRNA was aliquotted into the eight-well glass-based dishes (Nunc). Subsequently, HeLa cells (approximately 10 000 per well) were added to the wells. After incubation at 37 °C with 5% CO_2 for 72 h, the cells were immunostained with the antibodies as described in the text.

For the various assays that used kinase-knockdown cells, HeLa cells or HEK293 cells were plated on 35 mm dishes (Nunc) and transfected with 50 nM siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were analyzed after 72 h using an LSM510 laser scanning confocal microscope (Carl Zeiss Co., Ltd).

Quantification of Aβ

HEK-APP cells were plated on poly-L-lysine-coated six-well dishes (Sigma-Aldrich). After transfection of the siRNAs for 72 h, the cells were washed with PBS and then fresh medium was added. After a further incubation for 6 h, the medium and cells were collected. Quantification of Aβ in the medium was carried out by sandwich ELISA using a Human β Amyloid (1–40) ELISA Kit (Wako) and a Human β Amyloid (1–42) ELISA High Sensitive Kit (Wako), according to the manufacturer's instructions. The concentration of Aβ was normalized against the total protein concentration in the cell lysate as measured by the bicinchoninic acid (BCA) assay (Pierce).

Cholera Toxin Subunit B (CTxB) transport assay

After treatment with siRNA for 72 h, the cells were incubated with 1 μg/mL Alexa 556-conjugated CTxB (Alexa 556-CTxB) in PBS(+) (PBS with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) on ice for 30 min and then washed with PBS(+) to remove the unbound toxin. After incubation at 37 °C for various periods of time, the cells were washed and fixed. We counted the number of cells in which Alexa 556-CTxB had accumulated in the juxtanuclear region (N_j: juxtanuclear region-labeled cells), and then calculated the percentage of cells in which transport from the PM to the juxtanuclear region had occurred ($100 \times N_j$/total number of transfected cells).

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Validation of the efficiency of gene silencing by RNAi in our system.

Figure S2 The siRNA efficiency of gene silencing in HeLa cells or HEK-APP cells.

Figure S3 Subcellular localization of CI-M6PR in kinase-knockdown HeLa cells.

Figure S4 Effects of GSK3β or CLASP2 knockdown on the retrograde transport of Cholera toxin subunit B (CTxB) from the endosomes to the TGN.

Table S1 List of the kinase inhibitors and their incubation conditions

Table S2 List of the kinase siRNAs

Additional Supporting Information may be found in the online version of the article.

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A traditional medicinal herb *Paeonia suffruticosa* and its active constituent 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose have potent anti-aggregation effects on Alzheimer's amyloid β proteins *in vitro* and *in vivo*

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Abstract

The deposition of amyloid β (Aβ) protein is a consistent pathological hallmark of Alzheimer's disease (AD) brains; therefore, inhibition of Aβ fibril formation and destabilization of pre-formed Aβ fibrils is an attractive therapeutic and preventive strategy in the development of disease-modifying drugs for AD. This study demonstrated that *Paeonia suffruticosa*, a traditional medicinal herb, not only inhibited fibril formation of both $A\beta_{1-40}$ and $A\beta_{1-42}$ but it also destabilized pre-formed Aβ fibrils in a concentration-dependent manner. Memory function was examined using the passive-avoidance task followed by measurement of Aβ burden in the brains of Tg2576 transgenic mice. The herb improved long-term memory impairment in the transgenic mice and inhibited the accumulation of Aβ in the brain. Three-dimensional HPLC analysis revealed that a water extract of the herb contained several different chemical compounds including 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (PGG). No obvious adverse/toxic were found following treatment with PGG. As was observed with *Paeonia suffruticosa*, PGG alone inhibited Aβ fibril formation and destabilized pre-formed Aβ fibrils *in vitro* and *in vivo*. Our results suggest that both *Paeonia suffruticosa* and its active constituent PGG have strong inhibitory effects on formation of Aβ fibrils *in vitro* and *in vivo*. PGG is likely to be a safe and promising lead compound in the development of disease-modifying drugs to prevent and/or cure AD.

Keywords: 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose, Alzheimer's disease, amyloid β protein, medicinal herb, *Paeonia suffruticosa*, Tg2576 transgenic mice.

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGG, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose.

