厚生労働科学研究費補助金

認知症対策総合研究事業

新しいアッセイ法による認知症治療薬の効果判定

平成２３年度　総括研究報告書

研究代表者　二井　勇人

平成２４(2012)年　４月

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新しいアッセイ法による認知症治療薬の効果判定

総括研究者　　二井勇人　東北大学大学院　農学研究科　准教授

研究要旨
アルツハイマー病の原因となるアミロイドβ蛋白質（Aβ）の生成には、β、γセクレターゼの２つのプロテアーゼが関与する。私たちは、２つのセクレターゼを標的とした治療に向け、精製が困難で酵素機能が明らかになっていなかったヒトγセクレターゼ複合体を酵母において再構成し、試験管内でAβ産生活性を測定した。平成２３年度には、このAβ産生系を用いて、複合体内のサブユニット構成が活性に及ぼす影響、リン脂質やプロリン異性化酵素（Pin1）による活性の変化など、酵素としての基本的な性質を明らかにした。さらに、薬剤の開発に向け、天然物ライブラリーからγセクレターゼの活性を制御する薬剤をスクリーニングする一方、フェニルノスルタチン含有ペプチドを用いたβセクレターゼの阻害剤を解析している。

研究分担者：石浦章一
東京大学大学院・総合文化研究科・教授

A．研究目的

アルツハイマー病は、記憶障害・認知機能低下から患者のQOLを著しく低下させ死に追いやるだけでなく、看護する家族にも非常に大きな負担を強いる。しかしながら、効果的治療法は未だ開発されていない。本研究の目的は、アルツハイマー病の原因となるアミロイドβ蛋白質（Aβ）と呼ばれる42-43アミノ酸からなるペプチドの生成に関与するβ、γセクレターゼの酵素機能を解明し、私たちが明らかにした特徴から新たな治療法を提案することである。

Aβは、アミロイド前駆体蛋白質（APP）のプロテオリシスによって作られるが、これに主に関与するのがβ、γセクレターゼで、これらの阻害がアルツハイマー病征服の一番の標的である。私たちは精製が困難で、その酵素機能が依然として明らかになっていなかったヒトγセクレターゼ複合体を、酵母において再構成することに成功した。試験管内でγセクレターゼ活性を測定する系と、酵母の生育を指標にγセクレターゼ活性を評価するスクリーニング系を、世界で初めて開発した。これらの系を用いて、γセクレターゼの詳細な酵素学的検討や、γセクレターゼの活性を制御する薬剤や阻害剤を探索する。

また、私たちはβセクレターゼ（BACE1）についても、活性化機構の知見を積み重ねて、阻害剤を開発してきた。KMI化合物は、APPのβセクレターゼ切断部位をもとに人工アミノ酸フェニルノルスタチンを配してデザインされたBACE1阻害剤である。分担研究者の石浦の協力のもと、京都薬科大学の木曽良明教授にサンプルの提供を依頼し、フェニルノルスタチン化合物の添加による、βセクレターゼ活性の阻害を解析している。

B．研究方法

ヒトγセクレターゼを構成する４つの遺伝子（プレセニリン、ニカストリン、Aph-1、Pen2）と、ヒトアミロイド前駆体（APP）断片を酵母細胞内に導入し、その膜画分（ミクロソーム）を調製することにより、試験管内でAβを生成する事が可能になった。それによって、試験管内での反応条件を様々に変化させ、Aβの生成への影響を解析することができる。平成２２年度までの研究結果を受け、サブユニットのうちプレセニリン１とプレセニリン２の違いによるプロテアーゼ活性の変化、家族性アルツハイマー病プレセニリン変異体の活性と基質特異性（APP とNotch の比較）、アルツハイマー病脳内で減少するリン脂質（プラズマローゲン）やプロリン異性化酵素Pin1 の添加による活性への影響を解析した。

また、転写因子（Gal4）を融合した人工基質（APP-Gal4 や Notch-Gal4）を酵母に導入することによって、酵母の生育を指標にγセクレターゼ活性を

評価することができる。薬剤の添加によるγセクレターゼ活性と基質特異性の変化を、酵母の液体培地中での生育によって評価した。

さらに、APP のβ切断部位にフェニルノルスタチンを配した種々の新規KMI化合物によるAβ生成阻害活性を BACE1 安定発現細胞系を用いてスクリーニングした。

（倫理面への配慮）

ヒトアミロイド前駆体、BACE1、γセクレターゼ複合体の cDNA は、市販のライブラリーからクローニングしたものであり、家族性アルツハイマー病変異体は PCR 変異導入法により、新しく作製した。特定の相手方の同意・協力・人権及び利害の保護の取り扱いについては全く問題ない。

C．研究結果

平成２２年度からの研究を引き続きおこない、アルツハイマー病患者脳で減少するリン脂質、プラズマローゲンについてγセクレターゼ活性への影響を解析した。脳内には、ビニルエーテル結合を持つフォスファチジルエタノールアミン（PE）型プラズマローゲン（PEPls）が豊富に存在する。一般的なリン脂質である PE と比較して、ウシ由来 PEPls の効果を解析した結果、PEPls によってγセクレターゼ活性が阻害され産生Aβ量が減少した。

プロリン異性化酵素 Pin1 については、これまでAβ産生量を増加もしくは減少させると、正反対の結果が２つのグループから報告されていた。平成２２年度から引き続き、酵母Aβ産生系への精製 Pin1 添加の影響を解析した。その結果、Pin1 と APP の間の結合は確認されたものの、産生 Aβ総量への影響は見られなかった。しかし、産生 Aβ分子種が変化する（Aβ42 と Aβ43 が増加、Aβ40 が減少。数字はアミノ酸の長さ）ことが明らかとなった。

平成２２年度までに、プレセニリン１（PS1）とプレセニリン２（PS2）を酵母に導入して解析すると PS2 は PS1 に比べて活性が低いことが明らかとなった。この結果はこれまでの哺乳類細胞を用いた解析結果を再現するものであったが、さらにその原因について調べたところ、PS2 の低活性はγセクレターゼ複合体形成効率の悪さに起因するもので、複合体あたりの活性はほとんど変わらないことが明らかになった。また、家族性アルツハイマー病変異体（PS1, PS2）を導入したところ、Aβ生成活性は野生型に比べて著しく低いものであったが、Notch 切断への影響はあまり見られず、活性の低下には基質特異性があることが明らかとなった。

平成２２年度から継続して新規 KMI 化合物、非ペプチド性化合物を評価する一方、γセクレターゼ活性の制御を目標にして、天然物ライブラリー（東北大学内田隆史教授より供与）のスクリーニングを行っている。

D．考察

アルツハイマー病患者脳においてプラズマローゲンは有意に減少することが報告されている。本研究でγセクレターゼへの阻害活性が確認されたことにより、Aβ生産へのプラズマローゲンの直接的な関与が証明された。また、先行研究でプロリン異性化酵素 Pin1 欠損マウスの脳内ではアミロイド生成が上昇し、アミロイド斑を蓄積することが分かっていたが、γセクレターゼ活性への影響は解明されていなかった。本研究により、Pin1 がAβの切り分け機構に作用して、毒性・凝集性の高いAβ42 やAβ43 の生成の割合を変化させ、脳内でのアミロイド蓄積に関与していることが示唆された。プレセニリン２の低活性については、以前から議論があるところであったが、複合体形成の低下が原因となることが明らかとなった。今後、生体内では複合体形成効率を上げる因子があるのかについて興味が持たれる。

E．結論

酵母 Aβ生成系を用いて、γセクレターゼの酵素学的な性質を解明した。プラズマローゲンやプロリン異性化酵素 Pin1 との関連は、これまでには考えられていなかった全く新しい治療への方向性を生み出した。また、来年度以降γセクレターゼの活性に影響を与える薬剤を同定し、KMI 化合物との併用による培養細胞、モデルマウスの治療実験を行いたい。

F．健康危険情報

なし

なし

G．研究発表

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H．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（認知症対策総合研究事業）
分担研究報告書

γセクレターゼにおけるPS1とPS2の比較

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研究要旨
アスパラギン酸プロテアーゼの一種であるγセクレターゼは、アミロイドβ蛋白質（Aβ）生成の最終段階に関与する。γセクレターゼは４つのタンパク質からなる複合体であり、活性中心を担うプレセニリン（PS）には、PS1とPS2という２つのアイソフォームが存在する。私たちは、γセクレターゼホモログを持たない酵母を用いて、PS1のγセクレターゼとPS2のγセクレターゼを再構築し、活性や複合体形成について比較した。試験管内でAβ産生活性を測定したところ、PS2のγセクレターゼを含む膜画分の方が低い活性を示した。しかし、その原因について調べたところ、PS2の低活性はγセクレターゼ複合体形成効率の悪さに起因するもので、複合体あたりの活性はほとんど違わないことが明らかとなった。さらに、Aβ産生の至適pHが共にpH7.0付近であること、PS1とPS2のγセクレターゼ複合体では含まれるニカストリンの修飾が異なることが明らかとなった。

A．研究目的

アルツハイマー病の原因となるアミロイドβ蛋白質（Aβ）が生成する最終段階では、アミロイド前駆体蛋白質（APP）の断片が、アスパラギン酸プロテアーゼの一種であるγセクレターゼにより切断され、アミロイドβ蛋白質（Aβ）となる。γセクレターゼによる切断部位は複数箇所存在し、それぞれに相当したAβ分子種（Aβ40，Aβ42，Aβ43。数字はアミノ酸の長さ）が生成するが、このうちAβ42、Aβ43の毒性が高く、家族性アルツハイマー病でもこれらの分子種が蓄積する。γセクレターゼは４つのタンパク質（プレセニリン、ニカストリン、Aph-1、Pen2）からなる複合体であり、活性中心を担うプレセニリン（PS）には、PS1とPS2という２つのアイソフォームが存在する。PS1とPS2の活性の違いについては、過去に哺乳類培養細胞にそれぞれのPSを導入した解析から、PS2の方が、活性が低いとされてきたが、生化学的性質を厳密に比較した結果が無く、その原因は明らかになっていなかった。本研究では、PS1とPS2を含むγセクレターゼの活性、Aβ分子種、基質特異性、複合体形成を解析することを目的とした。

B．研究方法

γセクレターゼの４つの遺伝子と、ヒトアミロイド前駆体（APP）断片を酵母細胞内に導入し、PS1のγセクレターゼとPS2のγセクレターゼを再構築し、その膜画分（ミクロソーム）を用いて試験管内でAβ生成活性を測定した。また、転写因子（Gal4）を融合した人工基質（APP-Gal4やNotch-Gal4）を用いて、酵母の生育やレポーター遺伝子であるβガラクトシダーゼの酵素活性を指標に活性を評価し、PS1とPS2の基質特異性を比較した。

（倫理面への配慮）
ヒトアミロイド前駆体、γセクレターゼ複合体のcDNAは、市販のライブラリーからクローニングしたものであり、家族性アルツハイマー病変異体はPCR変異導入法により、新しく作製した。特定の相手方の同意・協力・人権及び利害の保護の取り扱いについては全く問題ない。

C．研究結果

プレセニリン１（PS1）とプレセニリン２（PS2）を酵母再構成系で解析するとPS2の方が、PS1に比べて活性が低いことが明らかとなった。これは、哺乳類細胞を用いた過去の解析結果を再現するものであったが、その原因を解明するために、免疫沈降法によってγセクレターゼ複合体を単離して複合体形成効率を測定した。その結果、PS2の低活性はγセクレターゼ複合体形成効率の悪さに起因するもので

あり、複合体あたりの活性はほとんど同じであることが明らかになった。また、PS1 と PS2 のγセクレターゼ複合体では含まれるニカストリンの修飾（糖鎖付加）が異なり、PS1 で見られる成熟型ニカストリンが PS2 のγセクレターゼでは見られず、未成熟型ニカストリンのみであった。Aβ生成の至適 pH は 7.0 であり、APP と Notch の基質特異性には、PS1 と PS2 で差は見られなかった。

一方、家族性アルツハイマー病プレセニリン（PS1, PS2）変異体を導入したところ、Aβ生成活性はいずれも野生型に比べて著しく低いものであった。一方、Notch 切断への影響はあまり見られず、活性の低下には基質特異性があることが明らかとなった。

D．考察

プレセニリン２については、哺乳類細胞を用いた遺伝子導入（大量発現）系での解析では、PS2 は PS1 に比べて活性が低いとされていた。今回、酵母再構成系を用いた解析でも同様の結果が得られたが、その原因として、PS2 を含むγセクレターゼの複合体形成能が低いということが明らかとなった。また、ゴルジ体以降でおこるニカストリンの糖鎖付加が PS2 を含むγセクレターゼで見られないことから、PS1 と PS2 のタンパク質輸送に違いがあることが考えられた。輸送と複合体形成の関連は今後検証しなければならない。また、生体内では複合体形成効率を上げる因子があるのかについて興味が持たれる。

E．結論

酵母 Aβ生成系を用いて、PS1 と PS2 の活性を比較し、酵素学的な性質、タンパク質輸送、複合体形成における違いを解明した。今後は、生成する Aβ分子種の違いに興味が持たれる。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

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研究成果の刊行に関する一覧表

雑誌

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IV. 研究成果の刊行物・別刷

Journal of Neuroscience Research

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Localization of Mature Neprilysin in Lipid Rafts

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Alzheimer's disease (AD) is characterized by senile plaques caused by amyloid-β peptide (Aβ) accumulation. It has been reported that Aβ generation and accumulation occur in membrane microdomains, called *lipid rafts*, which are enriched in cholesterol and glycosphingolipids. Moreover, the ablation of cholesterol metabolism has been implicated in AD. Neprilysin (NEP), a neutral endopeptidase, is one of the major Aβ-degrading enzymes in the brain. Activation of NEP is a possible therapeutic target. However, it remains unknown whether the activity of NEP is regulated by its association with lipid rafts. Here we show that only the mature form of NEP, which has been glycosylated in the Golgi, exists in lipid rafts, where it is directly associated with phosphatidylserine. Moreover, the localization of NEP in lipid rafts is enhanced by its dimerization, as shown using the NEP E403C homodimerization mutant. However, the protease activities of the mature form of NEP, as assessed by in vitro peptide hydrolysis, did not differ between lipid rafts and nonlipid rafts. We conclude that cholesterol and other lipids regulate the localization of mature NEP to lipid rafts, where the substrate Aβ accumulates but does not modulate the protease activity of NEP.

Key words: Alzheimer's disease; neprilysin; lipid rafts

Alzheimer's disease (AD) is characterized by the formation of senile plaques, composed primarily of amyloid-β peptide (Aβ). Aβ deposition has been thought to cause neurofibrillary tangles, neuronal cell loss, vascular damage, and dementia (the amyloid hypothesis; Hardy and Higgins, 1992). It has recently been suggested that AD begins with hippocampal synaptic dysfunction caused by diffusible oligomeric assemblies of Aβ (Selkoe, 2002).

Aβ is produced from amyloid precursor protein (APP) by the action of β- and γ-secretases, although APP is usually cleaved within the Aβ sequence by α-secretase. Aβ is degraded by neprilysin (NEP; Iwata et al., 2001). NEP is a type II membrane metallopeptidase that is capable of degrading not only monomeric Aβ but also pathological oligomeric Aβ (Kanemitsu et al., 2003). It has been reported that NEP levels in the hippocampus and cortex decline with age (Iwata et al., 2002; Hellstrom-Lindahl et al., 2008). Thus, analysis of the mechanisms regulating NEP activity may provide valuable insights for new therapeutic targets.

Recently, there have been several reports on the activities of proteases being regulated by their localization to membrane microdomains, known as *lipid rafts*. Lipid rafts, which are enriched in cholesterol and glycosphingolipids, have been implicated in processes such as signal transduction, endocytosis, and cholesterol trafficking (Pike, 2004, 2006). Whereas α-secretase cleavage occurs in nonlipid rafts (Kojro et al., 2001; von Tresckow et al., 2004), Aβ generation occurs in lipid rafts (Wada et al., 2003). It has been reported that Aβ accumulation is initiated by its association with GM1 in lipid rafts (Matsuzaki et al., 2007) and that NEP is partially localized in lipid rafts (Angelisova et al., 1999; Riemann et al., 2001; Kawarabayashi et al., 2004). However, whether the activity of NEP is regulated by its localization in lipid rafts is unknown.

Here we show that localization of glycosylated mature NEP in lipid rafts is regulated by its association with cholesterol. Moreover, we show with the NEP E403C homodimerization mutant that this localization is enhanced by its dimerization. Furthermore, we investigated the protease activities of mature NEP by an in vitro peptide assay. Unexpectedly, they were comparable in lipid rafts and nonlipid rafts. These findings suggest

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

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that cholesterol regulates the localization of mature NEP in lipid rafts, where the substrate Aβ accumulates but apparently does not modulate the protease activity of NEP.

MATERIALS AND METHODS

Vectors and Constructs

Human neprilysin, NEP WT, was inserted into the pcDNA3.1-3 × FLAG vector (Invitrogen, Carlsbad, CA), thereby fusing triplet tandem repeats of FLAG tag to its N-terminus. The expression product of this construct will be referred to as FLAG-NEP WT. NEP E584V, carrying a catalytically inactive mutant E584V, and NEP E403C, carrying a homodimerization mutant, were subcloned into the pcDNA3.1-3 × FLAG vector, yielding FLAG-NEP E584V and FLAG-NEP E403C, respectively.

Antibodies

The following antibodies were purchased: anti-FLAG M2 (Sigma, St. Louis, MO); antiflotillin-1 and anticalnexin (BD Transduction Laboratories, Lexington, KY); anti-monoclonal NEP (Leica Microsystems); and HRP-conjugated anti-mouse IgG (Cell Signaling Technology, Beverly, MA).

Cell Culture and Transfection

HEK293 cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma). They were maintained at 37°C in an atmosphere containing 5% CO_2 in a tissue culture incubator. DNA transfection was performed by lipofection with FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) when cells were 50% confluent. Then, 24 hr later, cells were harvested or used in assays.

Isolation of the Membrane Fraction

Cells were dissolved in TBS (0.1 M Tris-HCl, pH 8.0, 150 mM NaCl) containing Complete, EDTA-free protease inhibitor (Roche) and 0.7 μg/ml pepstatin A (Sigma) and disrupted by passage 20 times through a 21-G needle. The cell sample was then centrifuged (2,000 rpm, 2 min, 4°C). The resulting supernatant was then centrifuged again (49,000 rpm, 30 min, 4°C; Optima MAX-E ultracentrifuge; Beckman Coulter). The pellet formed was dissolved in TBS containing Complete, EDTA-free protease inhibitors, 0.7 μg/ml pepstatin A, and 1% Triton X-100; incubated on ice for 1 hr; and ultracentrifuged again. The resulting supernatant will be referred to as the *membrane fraction*.

Enzymatic Deglycosylation

The membrane fraction was solubilized with 1% Triton X-100 and then deglycosylated through treatment with the following: 1) endoglycosidase H (endo H; BioLabs), according to the manufacturer's instructions, and 2) 1 U N-glycosidase F (Endo F; Roche) per 45 μg of protein. The membrane fraction was denatured by boiling for 3 min in 1% SDS and 2-mercaptoethanol (ME), suspended in a reaction buffer (50 mM EDTA, 1% 2-ME, 0.5% Triton X-100, 0.1% SDS, 1 U N-glycosidase F) containing Complete, EDTA-free protease inhibitors and 0.7 μg/ml pepstatin A and incubated at 37°C overnight.

Isolation of Lipid Rafts by Sucrose Density Gradient Centrifugation

Cells were lysed on ice in MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% Triton X-100, Complete, EDTA-free protease inhibitors, and 0.7 μg/ml pepstatin A. Cell disruption was achieved by passing the lysate 10 times through a 21-G needle and then 20 times through a 27-G needle. The lysate was incubated at 4°C for 30 min, and an equal amount of 80% sucrose was then added to it. The sample and sucrose buffer, containing 5–40% sucrose, were sequentially loaded to the bottom of a tube and then centrifuged (36,000 rpm, 18 hr, 4°C; CP 70 WX ultracentrifuge; Hitachi). Fractions were collected from the top to the bottom. Equal volumes of these samples were analyzed by Western blotting.

Methyl-β-Cyclodextrin Treatment

HEK293 cells overexpressing FLAG NEP-WT were washed with PBS, treated with 10 mM methyl-β-cyclodextrin (MβCD; Trappsol) for 20 min in a CO_2 incubator at 37°C, and collected. Lipid rafts fractions were treated with 50 mM MβCD on ice for 1 hr, dissolved in a double volume of TBS containing Complete, EDTA-free protease inhibitors and 0.7 μg/ml pepstatin A, and centrifuged (49,000 rpm, 1 hr, 4°C). The supernatants were removed and the pellets dissolved in TBS.

Western Blotting

Equal amounts of protein samples were separated by SDS-PAGE or Blue Native-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA). In the case of Blue Native-PAGE, the membranes were washed and destained using methanol. The membranes were soaked in PBS containing 5% nonfat dried milk and 0.05% Tween for 1 hr and then incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.05% Tween, 0.1% BSA, and 1 mM NaN_3. After washing, the membranes were incubated with HRP-conjugated secondary antibody for 1 hr. Antigen–antibody complexes were detected by enhanced chemiluminescence using a LAS-3000 Luminescent Image Analyzer (Fujifilm). Signals were quantified in MultiGauge software (version 2.3; Fujifilm).

Assay of NEP-Dependent Neutral Endopeptidase Activity

NEP activity was measured in vitro by incubation at 37°C for 1 hr in 100 mM MES (pH 6.8) containing Complete, EDTA-free protease inhibitors, 10 μM Z-Leu-Leu-Leu-H, and as a substrate 50 μM Z-Ala-Ala-Leu-*p*-nitroanilide (ZALL-*p*-NA; Peptide Institute), in the presence or absence of 10 μM thiorphan, a specific inhibitor of NEP.

Interaction of NEP With Various Lipids

Lipid-spotted membrane (P-6002; Echelon Biosciences) was treated with TBS containing 1% skim milk and gently

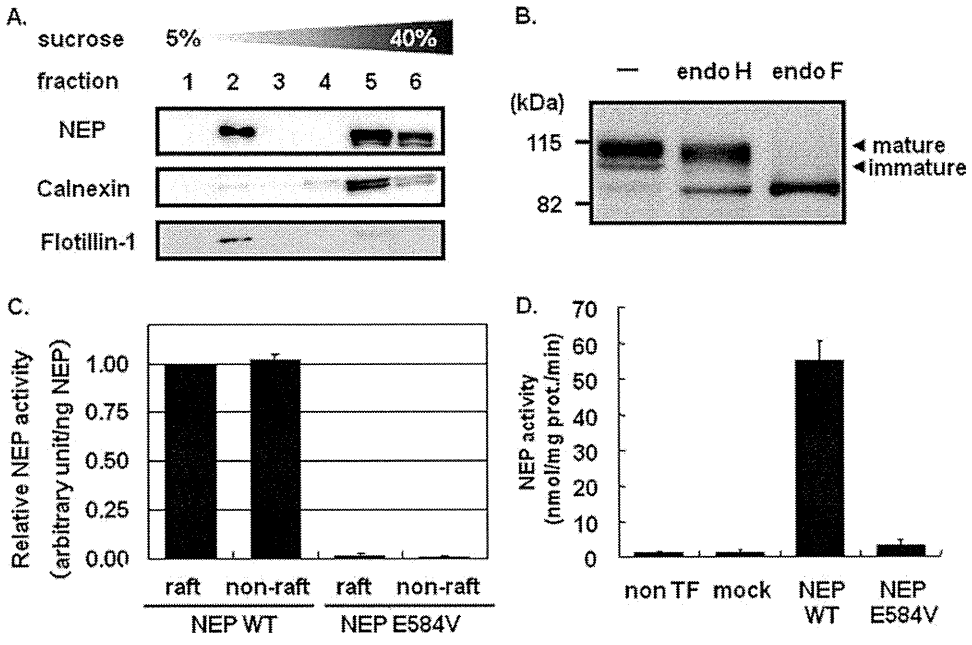

Fig. 1. NEP localization and activity in lipid rafts. **A:** Western blot analysis of lipid rafts fractionated from HEK293 cells overexpressing FLAG-NEP WT by a sucrose density-gradient centrifugation method. An anti-FLAG antibody was used to detect NEP. Lipid rafts were detected using an antibody raised against the raft marker flotillin-1. Nonlipid rafts (fraction 5) were detected using an antibody raised against the nonraft marker calnexin. **B:** Deglycosylation of the membrane fraction prepared from HEK293 cells overexpressing FLAG-NEP WT. The membrane fraction was treated with endoglycosidase H (endo H) and endoglycosidase F (endo F) or left untreated as a control (–), and then analyzed by Western blotting with an anti-FLAG antibody. **C:** Comparison of the specific enzymatic activity of the mature form NEP in lipid rafts (fraction 2) and nonlipid rafts (fraction 5), as assessed by *p*-NA peptide assay. Values represent the mean ± SD of three experiments. **D:** Neprilysin-dependent neutral endopeptidase activity in membrane fractions prepared from nontransfected HEK293 cells (non-TF) and cells transfected with vector (mock), FLAG-NEP WT (NEP WT) or the catalytically inactive mutant FLAG-NEP E584V. Values represent the mean ± SD of three experiments.

agitated for 1 hr at room temperature. SH-SY5Y neuronal cells were fractionated by sucrose density gradient centrifugation as shown previously, and each fraction was added to an equal volume of TBS containing protease inhibitor cocktail. After centrifugation at 49,000 rpm for 1 hr, the precipitate was dissolved in TBS containing protease inhibitor cocktail and incubated with the P-6002 membrane for 1 hr at room temperature. After incubation, the membrane was washed with TBS containing 0.1% Tween three times and incubated with anti-NEP monoclonal antibody diluted 1:2,000 for 1 hr at room temperature. The bound NEP was detected with an ECL advance kit (GE Healthcare, Amersham, United Kingdom).

RESULTS

Localization and Peptidase Activity of NEP in Lipid Rafts

To evaluate the peptidase activity of NEP in lipid rafts, we fractionated lipid rafts by sucrose density gradient centrifugation. We analyzed the localization of membrane-bound NEP extracted from HEK293 cells overexpressing FLAG-NEP WT. A raft marker, flotillin-1, was detected in fraction 2 and a nonraft marker, calnexin, in fractions 5 and 6 (Fig. 1A). FLAG-NEP was detected as a single band in fraction 2 and doublet bands in fractions 5 and 6. To distinguish these doublet bands, we deglycosylated the membrane fraction by treating it with endoglycosidase H (endo H) and endoglycosidase F (endo F; Fig. 1B). Although the upper band, the mature form, was resistant to endo H treatment, the lower band was deglycosylated by endo H. We will refer to the latter as the *immature form* of NEP. Resistance to endo H is acquired on transport of the protein to the Golgi apparatus, and this glycosylation is important for the catalytic activity of NEP (Lafrance et al., 1994). We compared the specific enzymatic activity of the mature form of NEP in lipid rafts (fraction 2) and nonlipid rafts (fraction 5); the contents of mature NEP were equalized by densitometric measurement of mature NEP levels after immunoblotting with an anti-FLAG antibody. The NEP activities of fractions 2 and 5, as assessed by *p*-NA peptide assay, were comparable (Fig. 1C). In this assay, catalytically inactive NEP E584V was used as a negative control (Fig. 1D).

Localization of NEP in Lipid Rafts Is Dependent on Cholesterol

Only mature NEP was detected in lipid rafts (Fig. 1A). We thus hypothesized that cholesterol in lipid rafts regulated the localization of mature NEP. To test this, we depleted HEK293 cells overexpressing FLAG-NEP

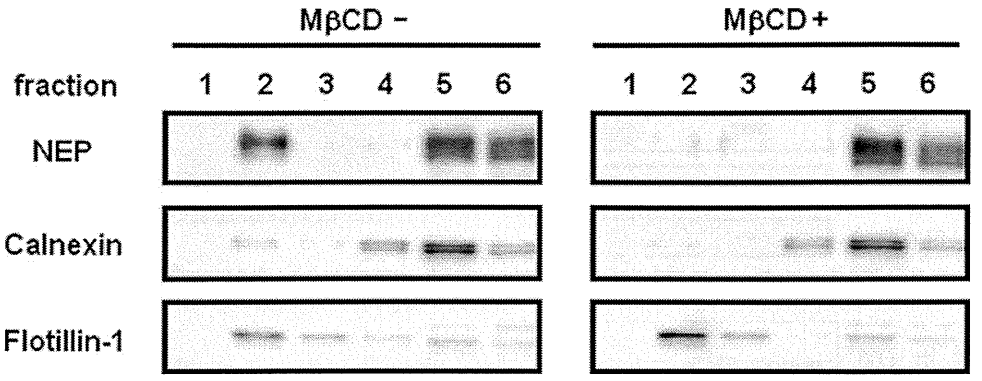

Fig. 2. Delocalization of NEP from lipid rafts in cells treated with MβCD. HEK293 cells overexpressing FLAG-NEP WT were treated with methyl-β-cyclodextrin (MβCD; +) or left untreated (–), and lipid rafts were fractionated as described in Materials and Methods.

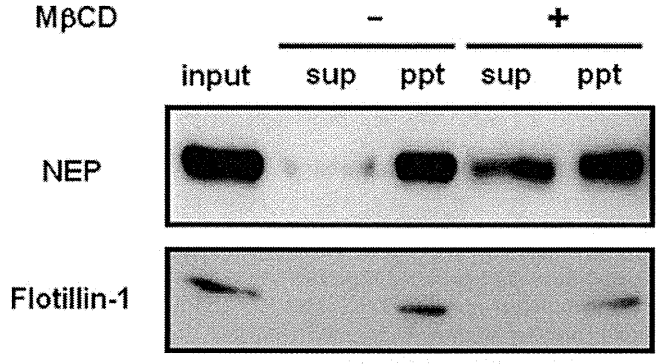

Fig. 3. Delocalization of NEP from fractionated lipid rafts after MβCD treatment. The lipid raft fraction, isolated from HEK293 cells overexpressing FLAG-NEP WT, was treated with (+) MβCD or left untreated (–) and separated into a supernatant (Sup) and a pellet (Ppt) by ultracentrifugation. The distribution of NEP was determined by Western blotting with an anti-FLAG antibody.

WT of cholesterol by treating them with 10 mM methyl-β-cyclodextrin (MβCD) for 20 min, and then fractionated the lipid rafts. More than 50% of cholesterol can be depleted from HEK293 cells by this treatment (Kojro et al., 2001). NEP became delocalized from lipid rafts following MβCD treatment, although flotillin-1 remained associated with them (Fig. 2).

We confirmed that the in vitro depletion of cholesterol from the lipid rafts fraction caused the delocalization of NEP from lipid rafts. We treated the fractionated lipid rafts with 50 mM MβCD for 1 hr at 4°C and separated them into supernatants and pellets by ultracentrifugation (Fig. 3). NEP and flotillin-1, associated with lipid rafts, were detected, as expected, in the pellets formed from lipid rafts not treated with MβCD. However, some of the NEP associated with lipid rafts was detected in supernatants prepared from lipid rafts treated with MβCD treatment. Flotillin-1 remained exclusively in the pellets, suggesting that flotillin-1 was not associated with choresterol.

Localization of NEP in Lipid Rafts Is Enhanced by Its Dimerization

To understand better the mechanism of NEP localization in lipid rafts, we investigated whether NEP dimerization facilitated the assembly of the enzyme in lipid rafts. We lysed HEK293 cells overexpressing FLAG-NEP WT in buffers containing different detergents and then analyzed NEP protein complexes by Blue Native-PAGE. Although NEP complexes were dissociated by NP-40 and Triton X-100, the 300-kDa complexes were resistant to treatment with DDM and digitonin (Fig. 4A). Next, we investigated the effect of dimerization on the localization of NEP in lipid rafts. It has been reported that rabbit NEP carrying an E403C mutation forms a covalent homodimer (Hoang et al., 1997). We introduced this mutation into human NEP and assessed its effect on the localization of NEP in lipid rafts. FLAG-NEP WT and FLAG-NEP E403C were detected as single 120-kDa bands after their separation by SDS-PAGE under reducing conditions (Fig. 4B). A 250-kDa FLAG-NEP E403C homodimer was detected under nonreducing conditions (Fig. 4B). These results indicate that, as in rabbit NEP, the E403C mutation caused human NEP to form of a covalent homodimer. Interestingly, although NEP WT complexes (Fig. 4A,C) were not resistant to Triton X-100, the NEP E403C mutant was resistant to Triton X-100 and formed a disulfide-bonded complex the same size as the NEP WT complex. Although we cannot exclude the possibility that the complex includes other proteins, the 300-kDa complex (Fig. 4A,C) appears to represent a covalent NEP homodimer.

Next, we compared the localization of mature forms of NEP WT and NEP E403C in lipid rafts. The ratio of the amount of mature NEP localized in lipid rafts to the total amount of mature NEP was 1.3 times higher in HEK293 cells overexpressing homodimeric mutant NEP E403C (47.7%) than in those expressing NEP WT (35.7%; Fig. 4D). These results demonstrate that the localization of NEP in lipid rafts was enhanced by its dimerization.

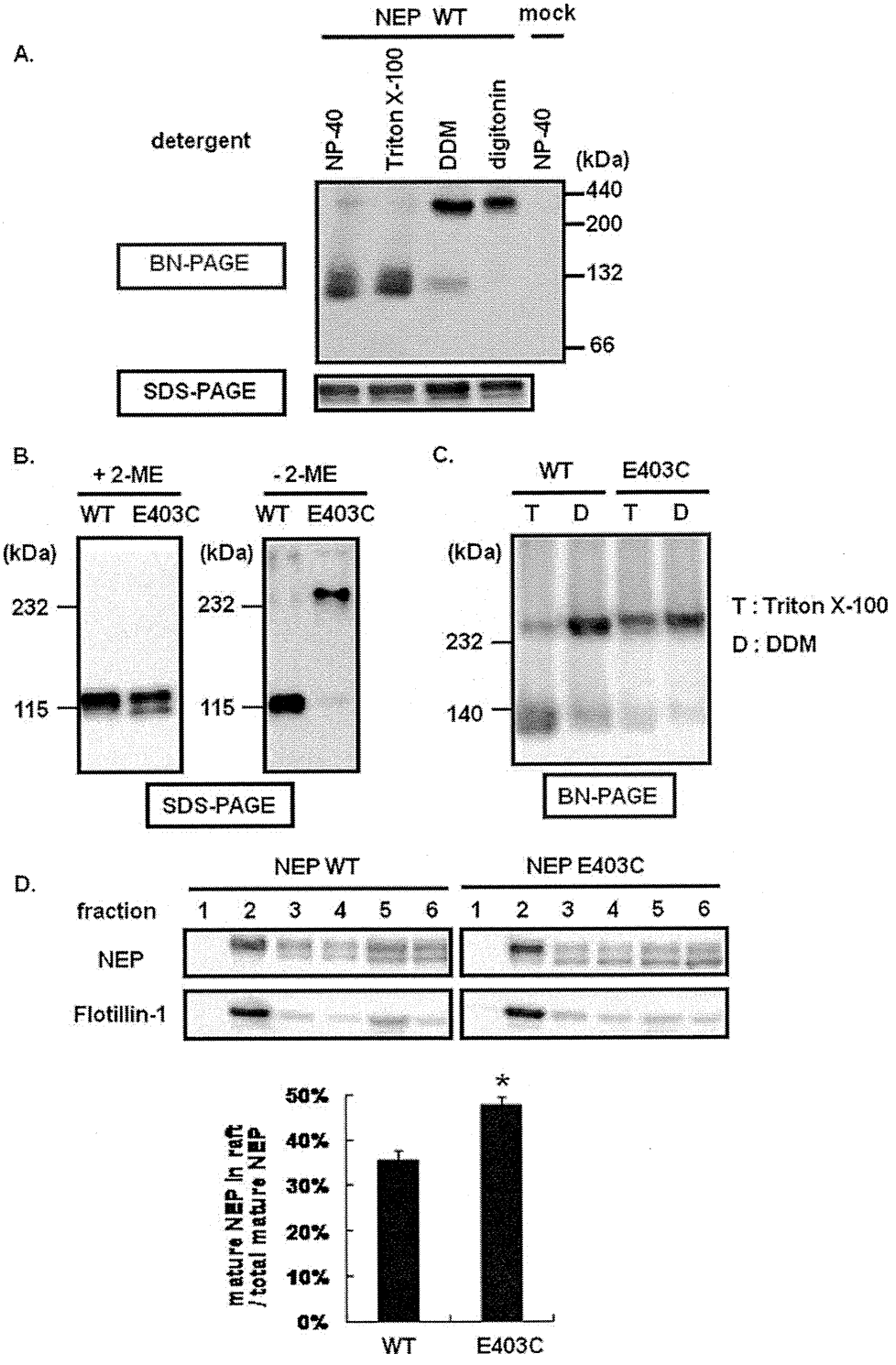

Fig. 4. Dimerization and localization of human NEP E403C in lipid rafts. **A:** Membrane fractions prepared from HEK293 cells overexpressing FLAG-NEP WT (NEP WT) or vector (mock) were dissolved in buffer containing detergents, such as NP-40, Triton X-100, DDM, and digitonin (all at a concentration of 1%). NEP complexes were analyzed by Blue Native-PAGE (BN-PAGE) or SDS-PAGE, followed by Western blotting with an anti-FLAG antibody. **B:** Membrane fractions obtained from HEK293 cells overexpressing FLAG-NEP WT (WT) or FLAG-NEP E403C (E403C) were analyzed by SDS-PAGE, performed with (**left**) or without (**right**) 2-ME. **C:** Membrane fractions obtained from HEK293 cells overexpressing FLAG-NEP WT (WT) or FLAG-NEP E403C (E403C) were dissolved in buffer containing 1% Triton X-100 (T) or 1% of DDM (D). The resulting lysates were analyzed by Blue Native-PAGE (BN-PAGE) and Western blotting with an anti-FLAG antibody. **D:** Effect of the E403C mutation on the distribution of NEP in lipid rafts. Lipid rafts from HEK293 cells overexpressing FLAG-NEP WT (NEP WT) or FLAG-NEP E403C (NEP E403C) were fractionated by sucrose density-gradient centrifugation and analyzed by Western blotting with an anti-FLAG antibody. The ratio of the amount of mature NEP localized in lipid rafts to the total amount of mature NEP was determined by densitometric measurement of protein bands corresponding to the mature form of NEP. Values represent the mean ± SD of three experiments. Statistical analysis was performed using a two-tailed Student's *t*-test. $\star P < 0.05$ was considered to indicate statistical significance (bottom graph).

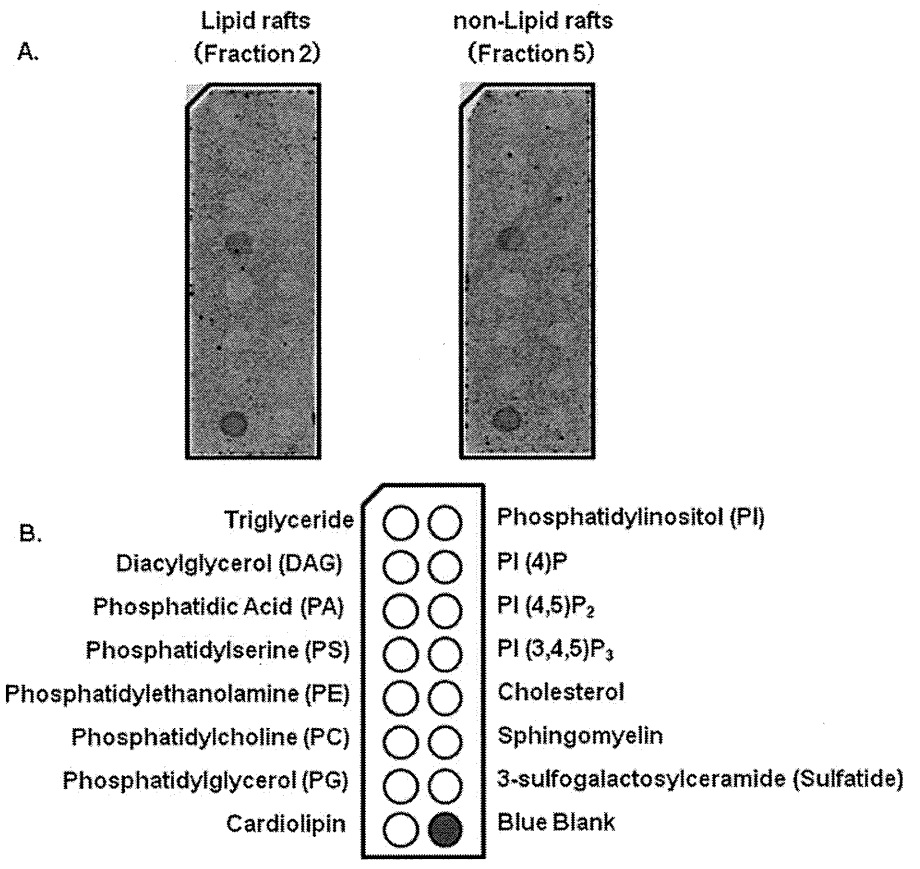

Fig. 5. Interaction of NEP with various lipids. Lipid-attached membrane was treated with sucrose density fractions of SH-SY5Y neuronal cells. After incubation and washing, the membrane was incubated with anti-NEP monoclonal antibody as described in Materials and Methods. The bound NEP was detected by ECL advance. **A:** ECL results. **B:** Lipids attached to the membrane.

Direct Inteaction of NEP With Lipids

The results described above suggest that NEP is localized in lipid rafts, possibly by its direct association with cholesterol. Finally, interaction of NEP with lipids was investigated by using lipid-spotted P-6002 membrane. Fractionated rafts (fraction 2 in Fig. 1A) and nonrafts fractions (fraction 5 in Fig. 1A) were concentrated by ultracentrifugation and incubated with lipids. After washing of the P-6002 membrane, lipid-bound NEP was detected by the specific antibody. Unexpectedly, NEP both in lipid rafts and in nonrafts fractions interacted with phosphatidylserine and cardiolipin but not with cholesterol (Fig. 5).

DISCUSSION

In this study, we found that only the mature form of NEP, glycosylated in the Golgi, and not the immature form, residing in the ER, was localized in lipid rafts (Fig. 1A,B). This indicates that complete glycosylation is required for the association of NEP with lipid rafts. Two possible explanations for this were considered. One is that maturation may be necessary for NEP to bind to a carrier protein such as a glycosylphosphatidylinositol (GPI)-anchored protein. The other is that a small conformational change caused by maturation increases the affinity of NEP for molecules found in lipid rafts, such as sphingolipids and cholesterol. With regard to the former, there have been several reports concerning carrier proteins. One study found that, when the transmembrane and C-terminal domains of BACE1 were replaced with a GPI anchor signal sequence, it was translocated to lipid rafts (Cordy et al., 2003). Another study found that the addition of the N-terminal domain of growth-associated protein 43 (GAP43) to the N-terminus of NEP increased the amount of NEP present in lipid rafts by 1.3-fold (Hama et al., 2004). With regard to the latter possible explanation, we found evidence that the localization of the mature form of NEP in lipid rafts was dependent on the content of cholesterol (Figs. 2, 3). Interestingly, although NEP was completely delocalized by cholesterol depletion, flotillin-1, a lipid raft marker, was not delocalized from lipid rafts by treatment with MβCD (Fig. 2). In this regard, flotillin-1 has been reported to be enriched in detergent-resistant microdomains that are MβCD resistant, although the mechanism

remains to be investigated (Rajendran et al., 2003). Moreover, to examine whether the delocalization of NEP from lipid rafts was caused by its direct association with cholesterol, we extracted lipid raft membranes and treated them with MβCD in vitro (Fig. 3). Consistently with the results presented in Figure 2, NEP was delocalized from lipid rafts membrane by cholesterol depletion, although not completely so (Fig. 3). The difference in the efficiency of NEP delocalization between cell and cell-free systems may be caused by the different conditions used (reaction temperature, membrane state, effects of ultracentrifugation). We conclude that the localization of mature NEP in lipid rafts depends on their cholesterol content.

We investigated the direct association of NEP with pure phospholipids and cholesterol (Fig. 5). Both NEP in rafts and nonrafts directly interacted with phosphatidylserine and cardiolipin. Cardiolipin is a major phospholipid of inner membrane of mammalian mitochondria, so phosphatidylserine might be the major interactor of NEP in lipid rafts. Moreover, immunocytochemical analysis showed that the clustered localization of endogenous NEP in SH-SY5Y cells became dispersed after MβCD treatment (Supp. Info. Fig. 1). Therefore, we conclude that NEP directly associated with phosphatidylserine in cholesterol-rich lipid rafts and MβCD-induced cholesterol depletion triggers the destruction of lipid composition and releases the NEP from rafts. However, the protease activities of mature NEP were unexpectedly comparable in lipid raft and nonlipid raft fractions, as assessed by *p*-NA peptide assay. It is possible that the fractionated lipid rafts did not reflect intracellular conditions (Pike, 2004). However, this result suggests that the association with lipid rafts does not itself modify the protease activity of NEP.

Considering the localization of Aβ in lipid rafts through association with cholesterol (Kakio et al., 2002), we hypothesized that the localization of mature NEP in lipid rafts facilitated its association with Aβ and thereby altered Aβ degradation. Recent studies have shown that lipid raft-dependent endocytosis is the predominant Aβ uptake mechanism (Lai and McLaurin, 2011), that there are correlations between memory deficits and intracellular Aβ levels in several mouse AD models (Billings et al., 2005; Knobloch et al., 2007; Bayer and Wirths, 2008), and that intracellular Aβ level correlates with extracellular amyloid deposition (Yang et al., 2011). Thus, it seems reasonable to conclude that NEP is localized and active in lipid rafts. Indeed, NEP is detected primarily in presynapses and on or around axons in the hippocampal formation (Fukami et al., 2002), and presynaptic NEP efficiently degrades Aβ (Iwata et al., 2004). Considering these findings, together with the fact that the ϵ4 allele of apolipoprotein E (apoE) is a risk factor in nonfamilial AD (Kim et al., 2009), we suggest that cholesterol, overloaded by aging or a high-fat diet, enlarges the area occupied by lipid rafts, thereby decreasing the likelihood of NEP and Aβ coming into contact with each other. As a result, Aβ becomes more abundant, oligomerizes, and causes memory deficits. However, it should be noted that cholesterol itself is a crucial contributor to synaptic structure and function. It has been reported that brain-derived neurotrophic factor (BDNF)-dependent cholesterol biosynthesis plays an important role in synapse development (Suzuki et al., 2007). It would therefore be important to maintain normal cholesterol metabolism during AD therapy.

We further investigated the effects of dimerization on the localization of NEP in lipid rafts. We introduced the E403C mutation into human NEP for the first time. The mutation was originally discovered in rabbit NEP, in which it causes the formation of a covalent homodimer (rabbit NEP normally exists as a monomer). Our results show that human NEP E403C, like rabbit NEP E403C, forms a covalent homodimer. In contrast, human NEP WT, like porcine NEP WT (Kenny et al., 1983), forms a noncovalent homodimer (Fig. 4A,B). Moreover, the noncovalent human NEP WT homodimer, though not resistant to NP-40 or Triton X-100, was resistant to DDM and digitonin. DDM and digitonin dissolve proteins modestly, so the complex remained intact after treatment with these detergents. Interestingly, the localization of mature NEP to lipid rafts was enhanced by its homodimerization (Fig. 4D). With regard to the endopeptidase activity of NEP E403C, Vmax/Km for this mutant was decreased by 50% compared with that for wild-type by using either [D-Ala^2, Leu^5] enkephalin or Suc-Ala-Ala-Leu-NH-Np as a substrate (Hoang et al., 1997). Although the NEP E403C mutant seems to be artificial and to have no physiological significance, these results imply that the protease activity of NEP might be modulated by its dimerization.

In conclusion, we have shown that cholesterol regulates the localization of mature NEP in lipid rafts, where its substrate, Aβ, accumulates. Cholesterol does not, however, modulate the protease activity of NEP.

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Comparison of Presenilin 1 and Presenilin 2 γ-Secretase Activities Using a Yeast Reconstitution System*[S]

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γ-Secretase is composed of at least four proteins, presenilin (PS), nicastrin (NCT), Aph1, and Pen2. PS is the catalytic subunit of the γ-secretase complex, having aspartic protease activity. PS has two homologs, namely, PS1 and PS2. To compare the activity of these complexes containing different PSs, we reconstituted them in yeast, which lacks γ-secretase homologs. Yeast cells were transformed with PS1 or PS2, NCT, Pen2, Aph1, and artificial substrate C55-Gal4p. After substrate cleavage, Gal4p translocates to the nucleus and activates transcription of the reporter genes *ADE2*, *HIS3*, and *lacZ*. γ-Secretase activity was measured based on yeast growth on selective media and β-galactosidase activity. PS1 γ-secretase was ~24-fold more active than PS2 γ-secretase in the β-galactosidase assay. Using yeast microsomes containing γ-secretase and C55, we compared the concentration of Aβ generated by PS1 or PS2 γ-secretase. PS1 γ-secretase produced ~24-fold more Aβ than PS2 γ-secretase. We found the optimal pH of Aβ production by PS2 to be 7.0, as for PS1, and that the PS2 complex included immature NCT, unlike the PS1 complex, which included mature NCT. In this study, we compared the activity of PS1 or PS2 per one γ-secretase complex. Co-immunoprecipitation experiments using yeast microsomes showed that PS1 concentrations in the γ-secretase complex were ~28 times higher than that of PS2. Our data suggest that the PS1 complex is only marginally less active than the PS2 complex in Aβ production.

γ-Secretase consists of at least four subunits, presenilin (PS),[3] nicastrin (NCT), anterior pharynx defective 1 (Aph1), and presenilin enhancer 2 (Pen2) (1). PS is the catalytic subunit of γ-secretase with aspartic protease activity (2, 3). Amyloid-β (Aβ) peptide, which plays a causative role in Alzheimer disease (AD), is produced after sequential cleavage of amyloid-β precursor protein (APP) by β-secretase and γ-secretase. The Aβ mainly consists of Aβ40 and Aβ42 containing 40 and 42 amino acids, respectively. Aβ42 is more prone to aggregation (4) and more toxic to neuronal cells. Many studies have reported that familial AD (FAD) mutations in PS and APP result in increased ratios of Aβ42 to Aβ40. The high Aβ42 ratio is believed to lead to AD.

PS has two homologs, namely, PS1 and PS2 (67% identical at the amino acid level). Aph1 also has two homologs: Aph1a (with alternative splicing variants Aph1a-S and a-L) and Aph1b. Sato *et al.* (5) reported that γ-secretase contained only one of each subunit, and as such, six distinct γ-secretases exist. Indeed, both PS1 and PS2 form a γ-secretase complex with the other subunits, producing Aβ (6). γ-Secretase cleaves many type I transmembrane proteins including APP and Notch, but the mechanism by which the different γ-secretases select their substrates is unclear. These different γ-secretases may have different functions and substrate selectivity.

Ubiquitous expression of PS1 and PS2 mRNAs in many human and mouse tissues has been reported, with varying expression levels across their tissues and during brain development (7). For example, in human young adult and aged brains, PS1 and PS2 mRNAs expression was similar. The subcellular distribution of PSs are known to be predominantly in the endoplasmic reticulum and the Golgi compartment (8). Levitan *et al.* (9) showed that human PS1 and PS2 substituted for *Caenorhabditis elegans* sel-12, suggesting that PS1 and PS2 are functionally redundant.

Different phenotypes of PS1- and PS2-deficient mice have been reported. PS1 knock-out mice exhibit severe developmental defects and perinatal lethality (10, 11), whereas PS2 knock-out mice show only mild phenotypes (12). Over 160 FAD mutations in PS1, but only 10 in PS2, have been found. These findings suggest that PS1 and PS2 play distinct roles *in vivo*.

Lai *et al.* (13) indicated that Ps1 (Ps, mouse presenilin) γ-secretase produced 169 times more Aβ than Ps2 γ-secretase, using membrane fractions from Ps1-(+/−), Ps2-(−/−), and Ps1-(−/−), Ps2-(+/+) blastocyst-derived cells from knock-out mice. In their study, γ-secretase activity was calculated as follows: level of produced Aβ/total Ps. They did not use the calculation: level of produced Aβ/Ps in γ-secretase complex and thus did not evaluate the active γ-secretase content.

Yagishita *et al.* (14) developed a novel γ-secretase assay using yeast microsomes. Yeast lacks endogenous γ-secretase and

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[3] The abbreviations used are: PS, presenilin; APP, amyloid precursor protein; Aβ, amyloid β peptide; Aph1, anterior pharynx 1; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CTF, carboxyl-terminal fragment; NCT, nicastrin; NTF, amino-terminal fragment; PC, phosphatidyl choline; Pen2, presenilin enhancer 2; FAD, familial Alzheimer disease; TM, transmembrane domain.