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２４年度　総括研究報告書

研究代表者　二井　勇人

平成２５(2013)年　５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産生活性を測定する酵素アッセイ系と、酵母の生育を指標にした活性評価（スクリーニング）系を構築した。平成２４年度には、これらの系を用いて天然物ライブラリーからγセクレターゼ阻害薬剤をスクリーニングする一方、アルツハイマー病の重篤度を決定するAβの切り分けに対するγセクレターゼモジュレーター【非ステロイド性抗炎症剤（NSAIDs）等】の作用機構を明らかにした。また、アルツハイマー病モデルマウス（Tg2576）の治療実験を行い、これまでに私たちが有効性を実証してきた経口Aβワクチン（Aβ米）とNSAIDsを投与したマウスにおいて、併用して治療することの有効性を示した。

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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。アルツハイマー病の原因は、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である。本研究の目的は、Aβの生成に関与する酵素の機能を解明し、私たちが明らかにした特徴から新たなアルツハイマー病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酵素アッセイ系と、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。これらの実験系を用いて、γセクレターゼ酵素学的性質を詳細に検討し、γセクレターゼ阻害剤を探索する。

一方、βセクレターゼ（BACE1）の活性化機構についても知見を積み重ねて、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阻害剤である。課題である細胞への透過性を向上させるために、京都薬科大学の木曽良明教授から提供していただいた新規化合物を用いて、βセクレターゼ活性の阻害を解析する。

また、私たちは、Aβを脳内から除去する方法として、食物ワクチン療法の有効性を検討してきた。Aβを発現させたピーマン葉や米をアルツハイマー病モデル（Tg2576）マウスに経口投与すると、脳内のAβ蓄積（老人斑形成）を防ぐことができる。本研究では、ワクチンと阻害剤の併用によるアルツハイマー病治療の有効性を解析する。

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。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２４年度は、この酵素アッセイ系を用いて、NSAIDs 添加に

よるγセクレターゼ活性への影響を解析した。また、ヒトAPP断片を発現させたチャイニーズハムスター卵巣（CHO）細胞の培地にNSAIDsを添加し、分泌するAβ分子種からNSAIDsの効果を解析した。

転写因子Gal4を融合した人工基質（APP-Gal4やNotch-Gal4）を酵母に導入すると、レポーター遺伝子の発現、即ち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。この系を用いて、天然物ライブラリー（東北大学内田隆史教授より供与）から薬剤探索を行った。酵母の生育で効果が見られた薬剤については、酵素アッセイ系を用いて試験管内での効果を解析した。

アルツハイマー病モデルマウスである、APPトランスジェニック（Tg2576）マウスにAβ発現米とNSAIDsを投与し（８～１１ヶ月齢）、その効果を、動物行動実験（Y迷路）により解析した。脳内での老人斑形成への効果については、免疫組織染色により解析した。

（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。動物実験は、東京大学動物実験センターからの承認、指導のもと、動物愛護に配慮して行った。

C．研究結果

第一に、γセクレターゼモジュレーター（NSAIDs）の作用機構を解析した。酵母Aβ生成系へのNSAIDs添加では、Aβ生成活性が著しく阻害された。また、APPを導入したチャイニーズハムスター卵巣（CHO）細胞の培地にNSAIDsを投与する実験を行ったところ、NSAIDsはAβ分子種のうち、毒性の高いAβ42（数字はアミノ酸長）の生成量を大きく下げることが明らかとなった。APPもしくはC99断片からは、Aβ40が９割程度、Aβ42が１割程度生成する。様々なAPP断片を検討した結果、C末端を欠失する断片では、Aβ42のみが生成する一方で、NSAIDsが効かなくなることが明らかとなった。

第二に、平成２３年度から継続して、γセクレターゼ活性の制御を目標にした天然物ライブラリーのスクリーニングを行った。スクリーニング総数1400のうち、57個を酵母の生育で選別し、実際にAβ生成系で阻害効果が見られた薬剤を１つ獲得した。スクリーニングによる問題点は、抗菌作用によって酵母が生育できない薬剤が多く、それによって多くの擬陽性が得られた。

最後に、アルツハイマー病モデルマウス（Tg2576）の治療実験を行った。私たちが開発した食物ワクチン（Aβ米）とNSAIDsを併せて経口投与する効果を解析した。１１ヶ月齢時で、自発行動量・空間作業記憶の低下に対して、併用による改善効果が見られている。Aβ米投与群では老人斑が除去されたが、併用によりそれが促進するかについてはさらに解析が必要である。

D．考察

γセクレターゼモジュレーター（NSAIDs）が、Aβ活性、なかでもAβ40とAβ42の切り分けに影響を与えるということが示唆されてきた。私たちの酵母を用いた解析系でもこれが確認できたことは大きな成果である。また、APPのC末端領域にNSAIDsが作用する可能性が示唆された。この領域は細胞質に露出した種々の蛋白質が結合する重要な領域であり、今後、詳細な作用機構を明らかにしたい。γセクレターゼ阻害剤のスクリーニングでは、酵母の生育を指標にしてスクリーニングするため、生育に対して影響を与える薬剤が擬陽性として取得された。今後は、レポーター遺伝子として酵素活性で評価するβ-ガラクトシダーゼ、α-ガラクトシダーゼを用いることを検討する。動物の治療実験では、これまでに効果が見られていたAβ米に加えてNSAIDsを投与するとさらに改善することが、行動実験で確認された。ワクチン療法と薬剤の併用については今後さらに検討していきたい。

E．結論

酵母Aβ生成系を用いて、γセクレターゼモジュレータNSAIDsの効果を検証し、APPのカルボキシ

末端領域が NSAIDs の作用に必要であることを明らかにした。カルボキシ末端領域の重要性はこれまでには全く考えられておらず、NSAIDs 作用機構の解明に向けて新しい方向性を生み出したといえる。また、モデルマウスの治療実験では、Aβ米を使った食物ワクチン療法と NSAIDs の併用の有効性を示した。

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

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セクレターゼモジュレーターの作用機構

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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。生成するAβ分子種のうち、Aβ42（数字はアミノ酸の長さ）は全体の約1割を占めるが、細胞への毒性が高くアルツハイマー病の発症を引き起こす。γセクレターゼモジュレーター（GSM）は、γセクレターゼの切断部位を変え、Aβ分子種の生成比率を変化させる薬剤である。特に、Aβ42を減少させるGSMは、アルツハイマー病の治療薬として期待されている。しかしながら、GSMの作用機構についてはよく分かっていない。私たちは、非ステロイド性抗炎症剤（NSAIDs）などAβ42を減少させるGSMについて、カルボキシ末端を欠損したアミロイド前駆体断片（CTF1-51とCTF1-52）への効果を検証した。はじめに、CTF1-51やCTF1-52からはAβ42のみが多量に生成し、γセクレターゼ活性のうちAβ42生成のみを解析できることが明らかになった。次に、NSAIDsの効果を検証したところ、通常の断片CTF1-99の切断に対してはAβ42の生成量を減少させるが、CTF1-51やCTF1-52にはほとんど効果がなかった。すなわち、アミロイド前駆体のカルボキシ末端にNSAIDsが作用することが示唆された。

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。通常はAβ40が約9割、Aβ42が約1割、Aβ43はごく少量生成するが、このうち毒性の高いAβ42、Aβ43の生成量が、家族性アルツハイマー病で増加することが知られている。γセクレターゼモジュレーター（GSM）は、Aβ分子種の組成を変える物質として発見された。Aβ42の量を減少させる化合物には、非ステロイド性抗炎症剤（NSAIDs）が知られている。しかしながら、その作用機構については、よく分かっていない。本研究では、酵母細胞にγセクレターゼを再構築したAβ生成系と、APPを発現させたチャイニーズハムスター卵巣（CHO）細胞を用いて、NSAIDsのγセクレターゼへの作用機構を明らかに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、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、ヒトAPP断片を発現させたチャイニーズハムスター卵巣（CHO）細胞の培地にNSAIDsを添加し、培養上清に分泌してくるAβ分子種を定量して、γセクレターゼ活性（Aβ切り分け）への影響を解析した。ヒトAPP断片には、通常の生体内に存在するCTF1-99に加え、カルボキシ末端領域の47アミノ酸もしくは48アミノ酸残基を欠損する断片（CTF1-51もしくはCTF1-52）を作製して使用した。

（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

酵母再構成系にNSAIDsを添加すると、Aβの生

成量が顕著に減少し、NSAIDs がγセクレターゼに阻害活性を示すことが明らかとなった。

一方、APP 断片を発現させた CHO 細胞では、NSAIDs の添加により分泌される Aβ総量にはあまり影響を受けなかったものの、Aβ42 の生成比率が著しく減少した。NSAIDs の作用機構についてさらに検討するために、APP のカルボキシ末端領域を欠いた断片（CTF1-51 もしくは CTF1-52）を保持する CHO 細胞を用いた実験を行ったところ、これらの断片からは Aβ40 はほとんど生成せず、通常は 1 割程度しか存在しない Aβ42 が大量に生成するということが明らかとなった。即ち、γセクレターゼが Aβ42 だけを生成する条件が得られたわけである。そこで、これらの断片の切断への NSAIDs の効果を解析した。その結果、予想外に NSAIDs の効果はほとんど見られなかった。

D．考察

酵母再構成系を用いた実験では、NSAIDs がγセクレターゼを阻害することが明らかとなった。このような阻害は哺乳類細胞を使った解析では観察されていない。酵母再構成系では、ミクロソーム膜を可溶化（1% CHAPSO）してから Aβを生成させるが、NSAIDs による阻害が試験管内での活性測定の条件によるのか、もしくは、酵母で再構成されたγセクレターゼ特有の性質か、今後の解析で明らかにしたい。

CHO 細胞を用いた実験では、APP のカルボキシ末端を欠く断片（CTF1-51 もしくは CTF1-52）からは Aβ42 だけが生成することが明らかとなった。これらの断片は APP の細胞質領域と膜貫通領域の３アミノ酸もしくは４アミノ酸分欠損している。基質の長さが切断部位の選択に関与することが示された。γセクレターゼの酵素機能を明らかにするうえで、重要な知見である。一方、これらの断片に対しては、NSAIDs が効果を示さないという予想外の結果が得られた。その原因はいくつか考えられるが、第一の可能性として、NSAIDs が作用する領域を失ったことが考えられる。すなわち、NSAIDs は APP のカルボキシ末端に作用することが示唆された。

E．結論

酵母γセクレターゼ再構成系を用いた実験と CHO 細胞を用いた実験から、NSAIDs の作用には、APP のカルボキシ末端が必要であることが示唆された。今後は、その可能性を直接的に証明するために NSAIDs の作用部位の特定を進めたい。

F．健康危険情報

なし

G．研究発表

１．論文発表

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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

雑誌

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Sato, K., Tanabe, C., Yoneyama, Y., Watahiki, H., Zhao, Y., Yagishita, S., Ebina, M., Suo, S., Futai, E., Murata, M. & Ishiura, S.	Localization of mature neprylysin in lipid rafts.	J. Neurosci. Res.	90	870-877	2012
Nagara, Y., Hagiyama, M., Hatano, N., Futai, E., Suo, S., Takaoka, Y., Murakami, Y., Ito, A. & Ishiura, S.	Tumor suppressor cell adhesion molecule 1 (CADM1) is cleaved by a disintegrin and metalloprotease 10 (ADAM10) and subsequently cleaved by γ -secretase complex.	Biochem. Biophys. Res. Commun.	417	462-467	2012
Hamada, Y., Nakanishi, T., Suzuki, K., Yamaguchi, R., Hamada, T., Hidaka, K., Ishiura, S. & Kiso, Y.	Novel BACE1 inhibitors processing a 5-nitroisophthalic scaffold at the P2 position.	Bioorg. Med. Chem. Lett.	22	4640-4644	2012
Hamada, Y., Tagad, H.D., Nishimura, Y., Ishiura, S. & Kiso, Y.	Tripeptidic BACE1 inhibitors devised by in-silico conformational structure-based design.	Bioorg. Med. Chem. Lett.	22	1130-1135	2012

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CTF1-51, a truncated carboxyl-terminal fragment of amyloid precursor protein, suppresses the effects of Aβ42-lowering γ-secretase modulators

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HIGHLIGHTS

- Cleavage of CTF1-51 was not suppressed by γ-secretase modulators.
- Treatment with GSMs did not significantly alter Aβ42 production from CTF1-51.
- CTF1-51 has some properties that reduce the effects of some γ-secretase modulators.

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ABSTRACT

The pathogenesis of Alzheimer's disease (AD) is correlated with the toxicity of amyloid β-peptide (Aβ), especially Aβ42. γ-Secretase modulators (GSMs) are compounds that alter production of Aβ42 without interfering with the physiological function of γ-secretase. Aβ42-lowering GSMs have been studied with the hope of using them as therapeutic or prophylactic drugs for AD. However, the mechanism of action of GSMs is not well defined. We examined the effect of Aβ42-lowering GSMs on model cells producing large amounts of Aβ42: CHO cells expressing CTF1-51, a precursor peptide of Aβ that is mainly cleaved into Aβ42. Our results indicate that the effect of GSM in the model was weak. Thus, we conclude that CTF1-51 cleavage mainly yields Aβ42 and suppresses the effects of some GSMs, a phenomenon that may be related to their mechanism of action.

1. Introduction

Progression of Alzheimer's disease (AD) is triggered by the aggregation and accumulation of amyloid β-peptide (Aβ) in the brain. It is therefore considered that prevention of the accumulation of Aβ will lead to the treatment or prophylaxis of AD. This assumption is called the amyloid hypothesis. Aβ is produced by the cleavage of amyloid precursor protein (APP), a type 1 transmembrane protein, by β-secretase and γ-secretase [14,17]. APP is cleaved by β-secretase into CTF1-99, a peptide that consists of the C-terminal 99 amino acids of APP. The transmembrane sequence of CTF1-99 is then cleaved by γ-secretase, yielding Aβ, a peptide that consists of 37–43 amino acids. Aβ forms aggregates and fibrils and promotes AD. Major molecular species of Aβ are Aβ40, which consists of 40 amino acids, and Aβ42, which consists of 42 amino acids. Aβ42 has higher aggregability and deposits in brain earlier than Aβ40 [5]. Thus, it is thought that reducing the production of Aβ42 will lead to the treatment or prophylaxis of AD.

γ-Secretase modulators (GSMs) are compounds that alter the ratio of molecular species of Aβ, and are classified into Aβ42-lowering GSMs and Aβ42-raising GSMs [7,18]. Aβ42-lowering GSMs could raise the production of Aβ38. GSMs could lower the production of Aβ42 without interfering with the physiological function of γ-secretase and may thus enable treatment or prophylaxis of AD without harmful side effects. However, the mechanism by which GSMs alter production of Aβ42 is not well-defined.

Here, we report that the effect of GSMs was decreased by application of CTF1-51, a truncated C-terminal fragment of APP, as an Aβ precursor peptide. CTF1-51 consists of the N-terminal 51 amino acids of CTF1-99, and γ-secretase mainly cleaves it into Aβ42 [3]. Thus, cells expressing CTF1-51 can be used as a model that produces a high ratio of Aβ42 to total Aβ. Studying the relationship between the effect of GSMs and an Aβ precursor peptide that mainly yields Aβ42 may contribute to elucidation of the mechanism of action of GSMs.

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2. Materials and methods

2.1. Reagents

Sulindac sulfide (Sigma–Aldrich, St. Louis, MO) and ibuprofen (Sigma–Aldrich) were used as Aβ42-lowering GSMs. L685,458 (Calbiochem, San Diego, CA) was used as a γ-secretase inhibitor. These compounds were dissolved in dimethyl sulfoxide (DMSO) and kept at −30 °C until use.

2.2. Cell culture

Stably-transfected Chinese hamster ovary (CHO) cells expressing human APP [6] and stably-transfected CHO cells expressing CTF1-51 [3] were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich) containing 10% fetal bovine serum, penicillin/streptomycin, and 200 μg/ml G418 (Calbiochem). CHO cells expressing CTF1-99 inducibly in the presence of 1 μg/ml tetracycline (Sigma–Aldrich) [12] were cultured in Nutrient Mixture F-12 Ham (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum, penicillin/streptomycin, 250 μg/ml Zeocin (Invitrogen, Carlsbad, CA), and 10 μg/ml Blasticidin S (Calbiochem). Cells were cultured in medium and treated for 24 h with L685,458 (1 μM), sulindac sulfide (60 μM), ibuprofen (250 μM), or vehicle (DMSO). Aβ in conditioned media was detected by Western blotting or sandwich ELISA.

2.3. Western blotting

For detection of Aβ by Western blotting, chloroform/methanol (2:1) was added to conditioned medium to extract lipids and to precipitate proteins. 45 min after the addition of chloroform/methanol, the tube was centrifuged for 15 min. After lipid extraction, the precipitated proteins were dissolved in sample buffer (2% SDS, 10% glycerol, 80 mM Tris–HCl, pH 6.8) and subjected to SDS-PAGE. To detect total amounts of Aβ, 16.5% polyacrylamide Tris/Tricine gels were used. To identify molecular species of Aβ, 10% polyacrylamide separation gels containing 8 M urea (pH 8.45) were used as previously described [12,19]. Proteins in gels were transferred onto a nitrocellulose membrane and Aβ was detected using the monoclonal antibody 82E1 (highly specific for the N-terminus of human Aβ) (IBL, Fujioka, Japan). The blots were visualized using an Enhanced Chemiluminescence system, and signal levels were quantified using an LAS-3000 luminescent image analyzer (Fuji Film, Tokyo, Japan).

2.4. ELISA

Levels of Aβ42, Aβ40, and Aβ38 in conditioned medium of CHO cells were measured using Aβ ELISA kits (IBL), according to the supplied instructions, and the absorbance of each well at 450 nm was measured using a Multiskan JX microplate reader (Thermo Fisher Scientific, Waltham, MA).

3. Results

3.1. Effects of a γ-secretase inhibitor and γ-secretase modulators on the cleavage of APP, CTF1-99, and CTF1-51 into Aβ

To evaluate the effect of L685,458, a transition state analog inhibitor of γ-secretase, and the Aβ42-lowering GSMs sulindac sulfide or ibuprofen, on production of Aβ by CHO cells expressing APP, CTF1-99, or CTF1-51, total Aβ secreted from CHO cells was detected by Western blotting (Fig. 1). Production of Aβ by all CHO cells was inhibited by L685,458. Sulindac sulfide and ibuprofen did not suppress the production of Aβ.

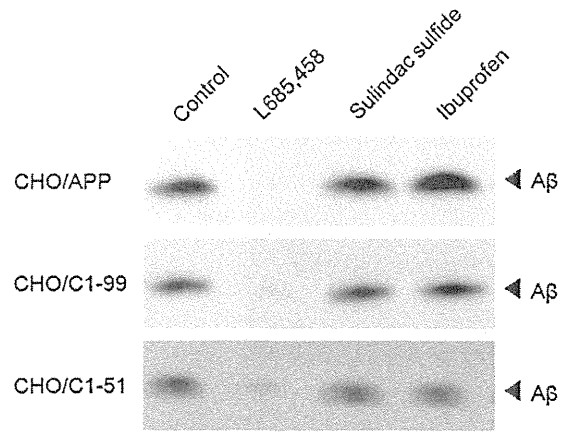

Fig. 1. Influence of a γ-secretase inhibitor and γ-secretase modulators on Aβ production in stably-transfected CHO cells expressing APP, CTF1-99, or CTF1-51. CHO cells were incubated with 1 μM L685,458, 60 μM sulindac sulfide, or 250 μM ibuprofen. After incubation for 24 h, the medium was concentrated and Aβ production was detected by Western blotting using 16.5% polyacrylamide Tris/Tricine gels and the monoclonal antibody 82E1.

3.2. Ratios of Aβ42 and Aβ40 produced by CHO cells treated with GSMs

Next, we analyzed the effect of GSMs on Aβ42 production by CHO cells expressing APP, CTF1-99, or CTF1-51. Aβ in conditioned medium was subjected to separation on 10% polyacrylamide gels containing 8 M urea (Fig. 2A, C, and E). In Fig. 2A, C, and E, Aβ species from Aβ37 to Aβ43 can be distinguished. To evaluate the effect of GSMs, Aβ42/Aβ40 ratios were compared (Fig. 2B, D, and F). Treatment with sulindac sulfide reduced the Aβ42/Aβ40 ratios of CHO cells expressing APP or CTF1-99, but not of CHO cells expressing CTF1-51. Ibuprofen did not significantly affect the Aβ42/Aβ40 ratios of CHO cells. We also calculated Aβ37/Aβ40 and Aβ43/Aβ40 ratios from Fig. 2. We found that Aβ37/Aβ40 ratio significantly increased, while Aβ43/Aβ40 ratio significantly decreased with ibuprofen treatment in CHO cells expressing CTF1-99 (both $p = 0.03$).

3.3. Amounts of Aβ42, Aβ40, and Aβ38 produced by CHO cells treated with GSMs

We also measured levels of Aβ species produced by CHO cells in conditioned medium. Conditioned medium was subjected to sandwich ELISA and production of Aβ42, Aβ40, and Aβ38 was measured (Fig. 3). When cells were treated with sulindac sulfide, the decrease in the production of Aβ42 in CHO cells expressing CTF1-51 was smaller than that in cells expressing APP. When cells were treated with ibuprofen, the increase in the production of Aβ38 in CHO cells expressing CTF1-51 was smaller than that in cells expressing APP.

4. Discussion

In this study, we found that the ability of a GSM (sulindac sulfide) to lower the production of Aβ42 in CHO cells expressing CTF1-51 was weaker than that in cells expressing APP or CTF1-99. Besides, the ability of another GSM (ibuprofen) to raise the production of Aβ38 in CHO cells expressing CTF1-51 was weaker than that in cells expressing APP. Thus, it is supposed that CTF1-51 has some properties that reduce the effects of some GSMs. This may be related to the mechanism of action of GSMs.

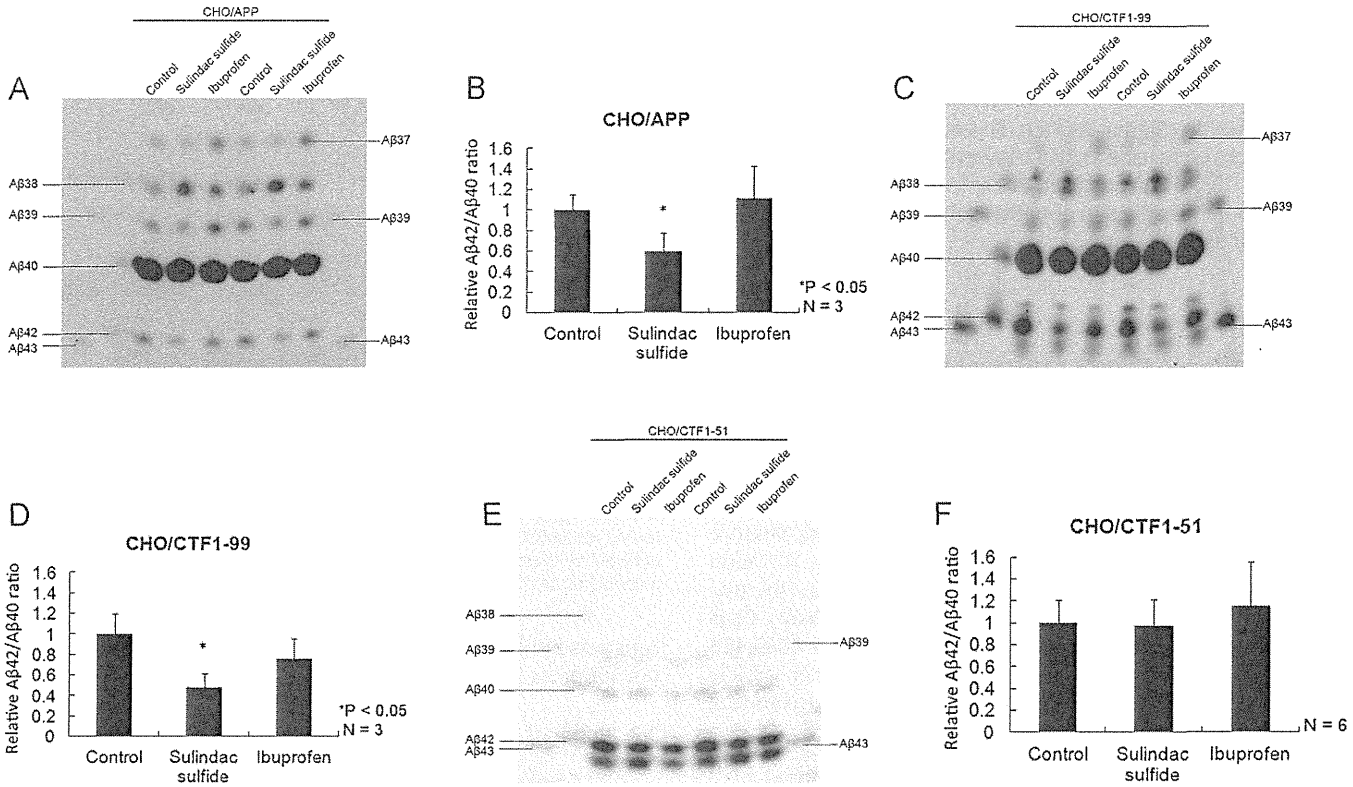

Fig. 2. Influence of GSMs on the Aβ42/Aβ40 ratio in stably-transfected CHO cells expressing APP (A and B), CTF1-99 (C and D), or CTF1-51 (E and F). (A, C, and E) CHO cells were incubated with 60 μM sulindac sulfide or 250 μM ibuprofen. After incubation for 24 h, the medium was concentrated and Aβ species were determined by Western blotting using 10% polyacrylamide separation gels containing 8 M urea and the monoclonal antibody 82E1. (B, D, and F) Relative Aβ42/Aβ40 ratio, compared with the control, was calculated from A, C, and E. $^*p < 0.05$ *vs.* control (two-tailed Student's *t*-test).

CTF1-51 has fewer C-terminal amino acids than the normal precursor peptide CTF1-99, and produces Aβ at a higher Aβ42/Aβ40 ratio. Although it is unclear how the properties of CTF1-51 influenced the effect of GSMs, we can suggest some hypotheses.

First, CTF1-51 may lack amino acid sequences that are important for the effects of GSMs. It has been shown that some GSMs, including sulindac sulfide and fenofibrate, bind to the Aβ region of the Aβ precursor peptide [8], and that GSMs such as GSM-1 and RO-57 bind to presenilin, the active component of γ-secretase [2,11]. However, the relationship between GSMs and the amino acid sequences of CTF1-99 other than in the Aβ region is unclear. CTF1-51 lacks the C-terminal 48 amino acids of CTF1-99. If the deletion of amino acids reduced the effect of GSMs, one could conclude that the missing amino acids are important.

Second, CHO cells expressing CTF1-51 produce a high ratio of Aβ42 to total Aβ. Thus, it is thought that CTF1-51 increases the Aβ42/Aβ40 ratio and may reduce the effects of Aβ42-lowering GSMs. With regard to this, there are two possible explanations. One is that CTF1-51 may affect the conformation of presenilin. Presenilin-1 mutations increasing the Aβ42/Aβ40 ratio change the conformation of presenilin-1, reducing the distance between the N- and C-termini [1]. Also, APP mutations increasing the Aβ42/Aβ40 ratio reduce the distance between the N- and C-termini of presenilin-1 [4], while APP mutations that reduce the Aβ42/Aβ40 ratio increase this distance [15]. Therefore, it is thought that production of Aβ42 is influenced by the conformation of presenilin, especially the distance between the N- and C-termini. Since CTF1-51 is a precursor peptide of Aβ that yields a high ratio of Aβ42 to total Aβ, it is thought that CTF1-51 may reduce the distance between the presenilin N- and C-termini. Other studies showed that Aβ42-lowering GSMs increase the distance between the N- and C-termini of presenilin, and that an Aβ42-raising GSM reduced this distance [9,16]. Accordingly, it is thought that GSMs alter the production of Aβ42 by changing the conformation of presenilin and that CTF1-51 may induce a conformational state that results in production of a high ratio of Aβ42 and thus reduces the effect of Aβ42-lowering GSMs.

Another possibility is that the homo-dimerization strength of the transmembrane sequences of CTF1-51 is related to the altered effects of GSMs. APP forms homo-dimers at transmembrane sequences and APP mutations decreasing the dimerization strength of the transmembrane sequences of APP reduce production of Aβ42 and increase Aβ38 levels [10]. Thus, CTF1-51 perhaps yields a high ratio of Aβ42 because of the high dimerization capacity of its transmembrane sequence. Other studies showed that Aβ42-lowering GSMs reduce the dimerization capacity of transmembrane sequences of an Aβ precursor peptide [13]. Accordingly, it is thought that GSMs alter the production of Aβ42 by changing the dimerization capacity of the transmembrane sequences of Aβ precursor peptides, and that the dimerization capacity of CTF1-51 may be stronger than that of APP, which may in turn reduce the effect of Aβ42-lowering GSMs.

The above explanations are not conclusive. It is possible that CTF1-51 lacks amino acid sequences that are important for the effect of GSMs, reduces the distance between the N- and C-termini of presenilin, and that its transmembrane sequences have a greater dimerization capacity than do those of APP. If the effect of GSMs is reduced in models that produce a high ratio of Aβ42, treatment of AD patients with GSMs under the condition that Aβ is produced at a high Aβ42/Aβ40 ratio in the brain may be unfavorable. To evaluate the efficacy of GSMs, studies on GSMs in models producing a high ratio of Aβ42 are important, and it will contribute to the development of new therapies for AD based on the amyloid hypothesis.

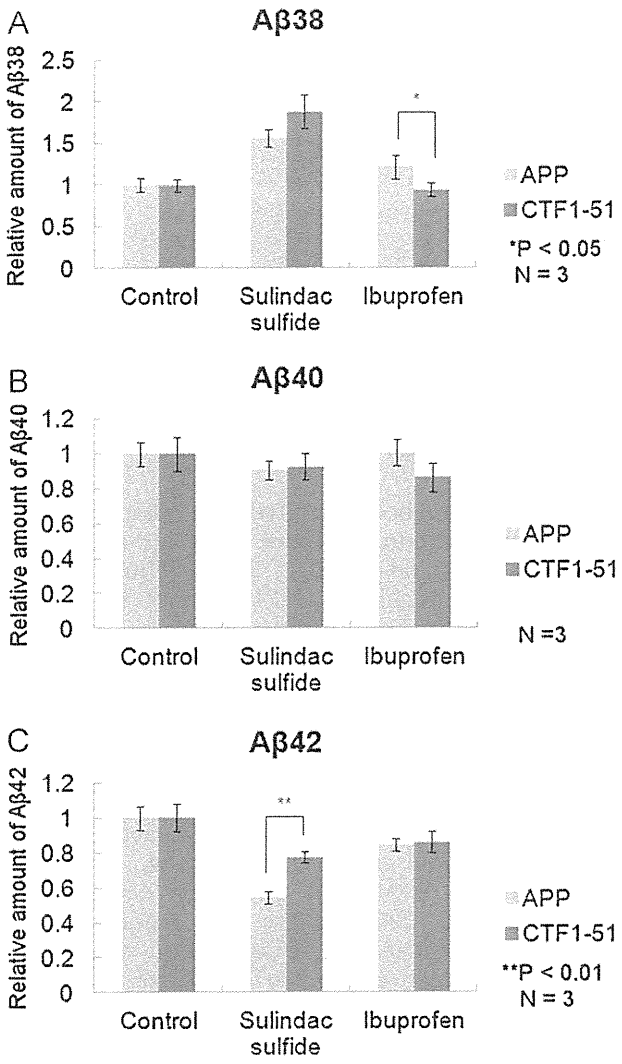

Fig. 3. Influence of GSMs on the production of Aβ42, Aβ40, and Aβ38 by stably-transfected CHO cells expressing APP or CTF1-51. CHO cells were incubated with 60 μM sulindac sulfide or 250 μM ibuprofen. After incubation for 24 h, the medium was subjected to sandwich ELISA to measure Aβ levels. All samples were measured in duplicate or triplicate. Relative Aβ levels were calculated compared to the control. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control (two-tailed Student's *t*-test).

We also found that Aβ37/Aβ40 ratio significantly increased, while Aβ43/Aβ40 ratio significantly decreased with ibuprofen treatment in CHO cells expressing CTF1-99 (Fig. 2C). On the other hand, sulindac sulfide did not alter these ratios significantly. These analyses indicated that treatment with ibuprofen can promote processing of Aβ43 to Aβ40 and then Aβ40 to Aβ37 in CHO cells expressing CTF1-99. This process from Aβ43 to Aβ37 should be confirmed in other systems in future.

Acknowledgments

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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.

K. Sato and C. Tanabe contributed equally to this work.

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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

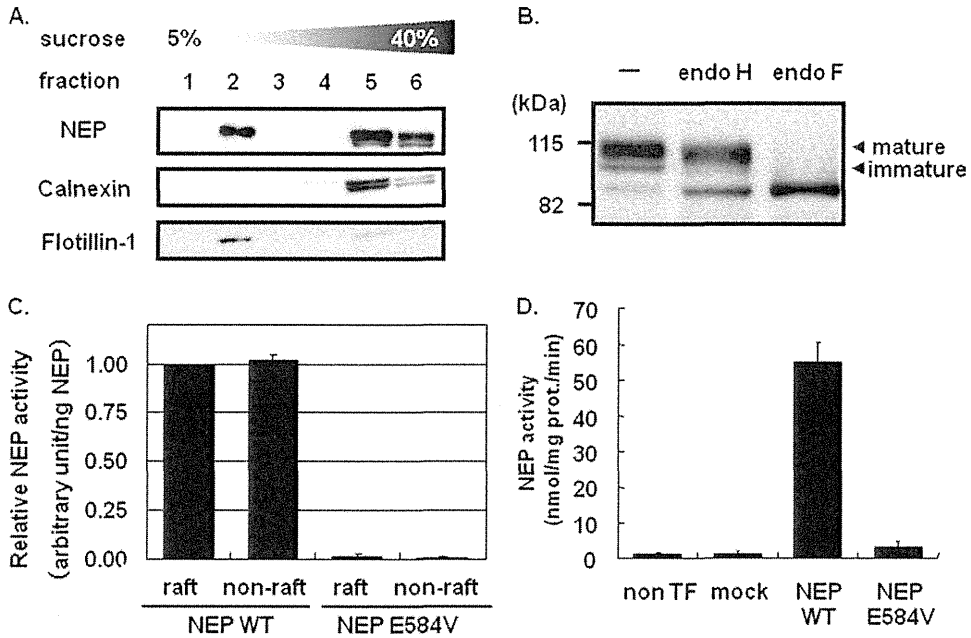

Fig. 1. NEP localization and activity in lipid rafts. **A:** Western blot analysis of lipid rafts fractionated from HEK293 cells overexpressing FLAG-NEP WT by a sucrose density-gradient centrifugation method. An anti-FLAG antibody was used to detect NEP. Lipid rafts were detected using an antibody raised 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

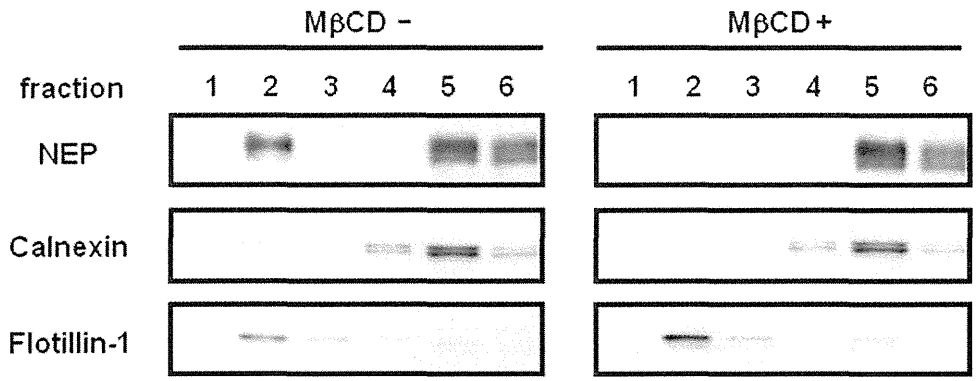

Fig. 2. Delocalization of NEP from lipid rafts in cells treated with MβCD. HEK293 cells overexpressing FLAG-NEP WT were treated with methyl-β-cyclodextrin (MβCD; +) or left untreated (–), and lipid rafts were fractionated as described in Materials and Methods.

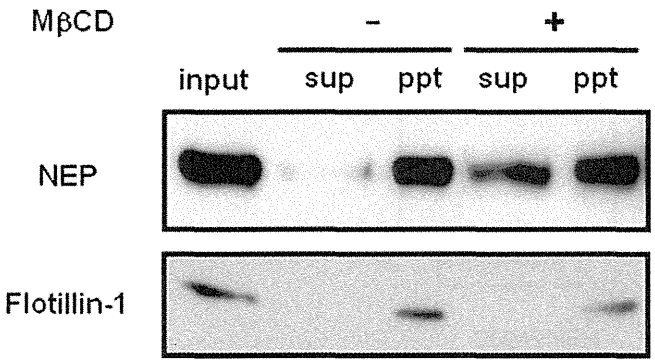

Fig. 3. Delocalization of NEP from fractionated lipid rafts after MβCD treatment. The lipid raft fraction, isolated from HEK293 cells overexpressing FLAG-NEP WT, was treated with (+) MβCD or left untreated (–) and separated into a supernatant (Sup) and a pellet (Ppt) by ultracentrifugation. The distribution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