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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22年度～24年度 総合研究報告書

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

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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①Aβ産生活性を測定する酵素アッセイ系と②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はじめて開発した。このうち酵素アッセイ系を用いて、複合体内のサブユニット構成が活性に及ぼす影響、金属、pH、リン脂質、非ステロイド性抗炎症剤（NSAIDs）など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セクレターゼ阻害剤をスクリーニングし新規阻害剤を取得した。一方、βセクレターゼ（BACE1）安定発現細胞系を用いて、フェニルノルスタチン含有ペプチド誘導體（新規KMI化合物、京都薬科大学木曾良明教授より分与）をスクリーニングし、ペプチド性薬剤の課題である脳内・細胞内への浸透性の向上が期待される薬剤を得ることに成功した。また、平成24年度には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明らかにした。

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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迫りやるだけでなく、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セクレターゼの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のペプチド配列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。課題である細胞への透過性を向上させるために、京都薬科大学の木曾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明らかにしてきた。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

(I) ヒト γ セクレターゼを構成する4つの遺伝子(プレセニン、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A β を生成する事が可能になった。この酵素アッセイ系を用いて、試験管内での反応条件【pH条件、金属イオンや薬剤(NSAIDsなど)の添加】を様々に変化させ、A β 生成への影響を解析する。また、 γ セクレターゼのサブユニットのうち、触媒サブユニットであるプレセニンアイソフォーム(PS1とPS2)、家族性アルツハイマー病家系から見つかったプレセニン変異体、Aph-1アイソフォーム(Aph-1aとAph-1b)を導入して、各々の複合体のプロテアーゼ活性と基質特異性を比較、解析する。

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A β 分子種からNSAIDsの効果を解析した。

(II) 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、すなわ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。

(III) 新規KMI化合物の探索では、BACE1を安定に発現させたヒト胚性腎臓(HEK293)細胞(BACE1安定発現細胞系)の培地に化合物を添加し、APPが切断されて培地に分泌されるsAPP β の量からBACE1阻害活性を算出した。

(IV) 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A β 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

(I-1) γ セクレターゼの至適活性条件、基質特異性(APPとNotchの切断)など、酵素としての基本的な性質を解析した。その結果、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よって、 γ セクレターゼが阻害されることがわかった。また、プロリン異性化酵素Pin1により、産生A β 分子種が変化する(A β 42とA β 43が増加、A β 40が減少。数字はアミノ酸の長さ)ことを明らかにした。

(I-2) γ セクレターゼ複合体のサブユニットを交換した結果、①プレセニン2(PS2)の活性は、プレセニン1(PS1)と比較して著しく低い(10分の1程度)こと、②Aph1のアイソフォーム間では活性に差がないことが明らかになった。PS2の低活性について原因をさらに調べたところ、 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、③家族性アルツハイマー病プレセニン変異体を導入したところ、A β 生成活性が著しく低くなる一方で、Notch切断は野生型とあまり変わらず、切断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。

(I-3) 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A β 生成系へのNSAIDs添加では、A β 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A β 分子種のうち、毒性の高いA β 42の生成量を大きく下げることが明らかとなった。APPからは、A β 40が9割程度、A β 42が1割程度生成する。様々なAPP断片から生成するA β 分子種を解析した結果、C末端を欠失する断片では、A β 42

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。

(II) 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)から γ セクレターゼ阻害剤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 A β 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。

(III) BACE1 安定発現細胞系を用いた BACE1 阻害剤の探索では、添加濃度 100 μ M でのスクリーニングの結果、ペプチド類似構造の KMI 化合物 KMI-714、-725 において、20%以下の強い阻害活性が認められた。また低濃度 (30 μ M) での解析の結果、50%以下の阻害活性を示し従来の化合物に準ずる活性を有する KMI-714、-725、-1027、-1036 を見出した。また、細胞への透過性を考えてデザインされた非ペプチド構造の化合物 (KMI-1023、-1027、-1030、-1036) では、試験管内での酵素阻害活性の強度にほぼ相関して培養細胞系での阻害活性が出る事が分かった。

(IV) アルツハイマー病モデルマウス (Tg2576) の治療実験では、私たちが開発した食物ワクチン (A β 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た。A β 米投与群では老人斑の除去機構が得られたが、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

(I-1) 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、発症における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、老人斑を蓄積することが分かっていた。本研究により、Pin1 が A β の切り分けに作用して、毒性の高い A β 42 の生成の割合を増加させ、アミロイド蓄積に関与していることが示唆された。

(I-2) 哺乳類細胞を用いた大量発現系では、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、PS2 の複合体形成能が低いことがその原因と考えられた。PS1 と PS2 のタンパク質輸送に違いがあることを示す結果も得ており、タンパク輸送と γ セクレターゼ複合体形成の関連を今後検証する。家族性アルツハイマー病変異体で A β 生成活性が減少したが、A β 総量は減少する一方、毒性の高い A β 42 の割合は上昇しており、活性の低下と A β 切り分けとの関連が興味深い。

(I-3) 酵母再構成系を用いた実験では、NSAIDs による γ セクレターゼの阻害が明らかとなった。このような阻害は哺乳類細胞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 A β 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場合によるのか、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 A β 42 だけが生成することが明らかとなった。基質の長さが切断部位の選択に関与することを示す重要な知見である。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結果が得られた。NSAIDs が APP の C 末端領域に作用することが示唆された。

(II) 酵母の生育を指標にした γ セクレターゼ阻害剤のスクリーニングでは、生育に対して影響を与える薬剤が多く擬陽性となってしまった。今後は、レポーター遺伝子の内、酵素活性を測定できる β -ガラクトシダーゼ、 α -ガラクトシダーゼを評価に加えることを検討する。

(III) 薬物動態に改良を加えた β セクレターゼ阻害薬、新規 KMI 化合物を同定したことは、治療を考える上で、大きな前進である。

(IV) アルツハイマー病モデルマウス (Tg2576) の治療実験で効果が見られていた A β 米に加えて、NSAIDs を投与するとさらに病態が改善することが明らかになった。ワクチン療法と NSAIDs を併用することの有効性は今後さらに確認していきたい。

E. 結論

酵母再構成系を用いて、 γ セクレターゼの酵素学的な性質を解明した。なかでも、プラズマローゲンと γ セクレターゼの関連は、これまでには考えられていなかった全く新しい治療法開発への方向性を生み出した。一方、BACE1阻害剤（新規KMI化合物）の改良、 $A\beta$ 米とNSAIDsの併用によるモデルマウスの治療効果の促進など、アルツハイマー病治療に直結する成果をあげた。

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

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Watahiki, H., Yagishita, S., Futai, E. & Ishiura, S.	CTF1-51, a truncated carboxyl-terminal fragment of amyloid precursor protein, suppresses the effects of A β -lowering γ -secretase modulators.	Neurosci. Lett.	526	96-99	2012
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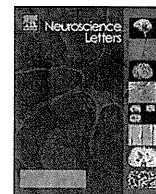
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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
二井勇人	サーチュインSIRT6は老化を防ぐのか？- その仕組みの解明に向けて	遺伝	66	478-481	2012
二井勇人	記憶力を良くしたい！- インスリン様成長因子（IGF-II）のはたらき	遺伝	65	100-104	2011
二井勇人、石浦章一	膜結合型メタロプロテアーゼ（MMP, ADAM）ファミリー	脳 2 1	13	33-38	2010

IV. 研究成果の刊行物・別刷



CTF1-51, a truncated carboxyl-terminal fragment of amyloid precursor protein, suppresses the effects of A β 42-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 A β 42 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 (A β), especially A β 42. γ -Secretase modulators (GSMs) are compounds that alter production of A β 42 without interfering with the physiological function of γ -secretase. A β 42-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 A β 42-lowering GSMs on model cells producing large amounts of A β 42: CHO cells expressing CTF1-51, a precursor peptide of A β that is mainly cleaved into A β 42. Our results indicate that the effect of GSM in the model was weak. Thus, we conclude that CTF1-51 cleavage mainly yields A β 42 and suppresses the effects of some GSMs, a phenomenon that may be related to their mechanism of action.

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1. Introduction

Progression of Alzheimer's disease (AD) is triggered by the aggregation and accumulation of amyloid β -peptide (A β) in the brain. It is therefore considered that prevention of the accumulation of A β will lead to the treatment or prophylaxis of AD. This assumption is called the amyloid hypothesis. A β 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 A β , a peptide that consists of 37–43 amino acids. A β forms aggregates and fibrils and promotes AD. Major molecular species of A β are A β 40,

which consists of 40 amino acids, and A β 42, which consists of 42 amino acids. A β 42 has higher aggregability and deposits in brain earlier than A β 40 [5]. Thus, it is thought that reducing the production of A β 42 will lead to the treatment or prophylaxis of AD.

γ -Secretase modulators (GSMs) are compounds that alter the ratio of molecular species of A β , and are classified into A β 42-lowering GSMs and A β 42-raising GSMs [7,18]. A β 42-lowering GSMs could raise the production of A β 38. GSMs could lower the production of A β 42 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 A β 42 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 A β precursor peptide. CTF1-51 consists of the N-terminal 51 amino acids of CTF1-99, and γ -secretase mainly cleaves it into A β 42 [3]. Thus, cells expressing CTF1-51 can be used as a model that produces a high ratio of A β 42 to total A β . Studying the relationship between the effect of GSMs and an A β precursor peptide that mainly yields A β 42 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 A β 42-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 $\mu\text{g}/\text{ml}$ G418 (Calbiochem). CHO cells expressing CTF1-99 inducibly in the presence of 1 $\mu\text{g}/\text{ml}$ tetracycline (Sigma–Aldrich) [12] were cultured in Nutrient Mixture F-12 Ham (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum, penicillin/streptomycin, 250 $\mu\text{g}/\text{ml}$ Zeocin (Invitrogen, Carlsbad, CA), and 10 $\mu\text{g}/\text{ml}$ Blastocidin 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). A β in conditioned media was detected by Western blotting or sandwich ELISA.

2.3. Western blotting

For detection of A β 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 A β , 16.5% polyacrylamide Tris/Tricine gels were used. To identify molecular species of A β , 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 A β was detected using the monoclonal antibody 82E1 (highly specific for the N-terminus of human A β) (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 A β 42, A β 40, and A β 38 in conditioned medium of CHO cells were measured using A β 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 A β

To evaluate the effect of L685,458, a transition state analog inhibitor of γ -secretase, and the A β 42-lowering GSMs sulindac sulfide or ibuprofen, on production of A β by CHO cells expressing APP, CTF1-99, or CTF1-51, total A β secreted from CHO cells was detected by Western blotting (Fig. 1). Production of A β by all CHO

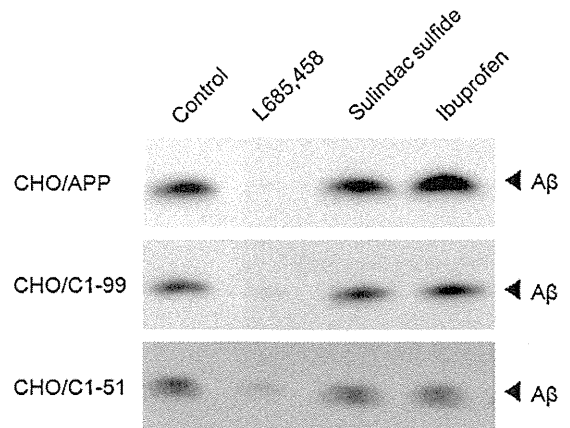


Fig. 1. Influence of a γ -secretase inhibitor and γ -secretase modulators on A β production in stably-transfected CHO cells expressing APP, CTF1-99, or CTF1-51. CHO cells were incubated with 1 μM L685,458, 60 μM sulindac sulfide, or 250 μM ibuprofen. After incubation for 24 h, the medium was concentrated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

cells was inhibited by L685,458. Sulindac sulfide and ibuprofen did not suppress the production of A β .

3.2. Ratios of A β 42 and A β 40 produced by CHO cells treated with GSMs

Next, we analyzed the effect of GSMs on A β 42 production by CHO cells expressing APP, CTF1-99, or CTF1-51. A β 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, A β species from A β 37 to A β 43 can be distinguished. To evaluate the effect of GSMs, A β 42/A β 40 ratios were compared (Fig. 2B, D, and F). Treatment with sulindac sulfide reduced the A β 42/A β 40 ratios of CHO cells expressing APP or CTF1-99, but not of CHO cells expressing CTF1-51. Ibuprofen did not significantly affect the A β 42/A β 40 ratios of CHO cells. We also calculated A β 37/A β 40 and A β 43/A β 40 ratios from Fig. 2. We found that A β 37/A β 40 ratio significantly increased, while A β 43/A β 40 ratio significantly decreased with ibuprofen treatment in CHO cells expressing CTF1-99 (both $p=0.03$).

3.3. Amounts of A β 42, A β 40, and A β 38 produced by CHO cells treated with GSMs

We also measured levels of A β species produced by CHO cells in conditioned medium. Conditioned medium was subjected to sandwich ELISA and production of A β 42, A β 40, and A β 38 was measured (Fig. 3). When cells were treated with sulindac sulfide, the decrease in the production of A β 42 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 A β 38 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 A β 42 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 A β 38 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.

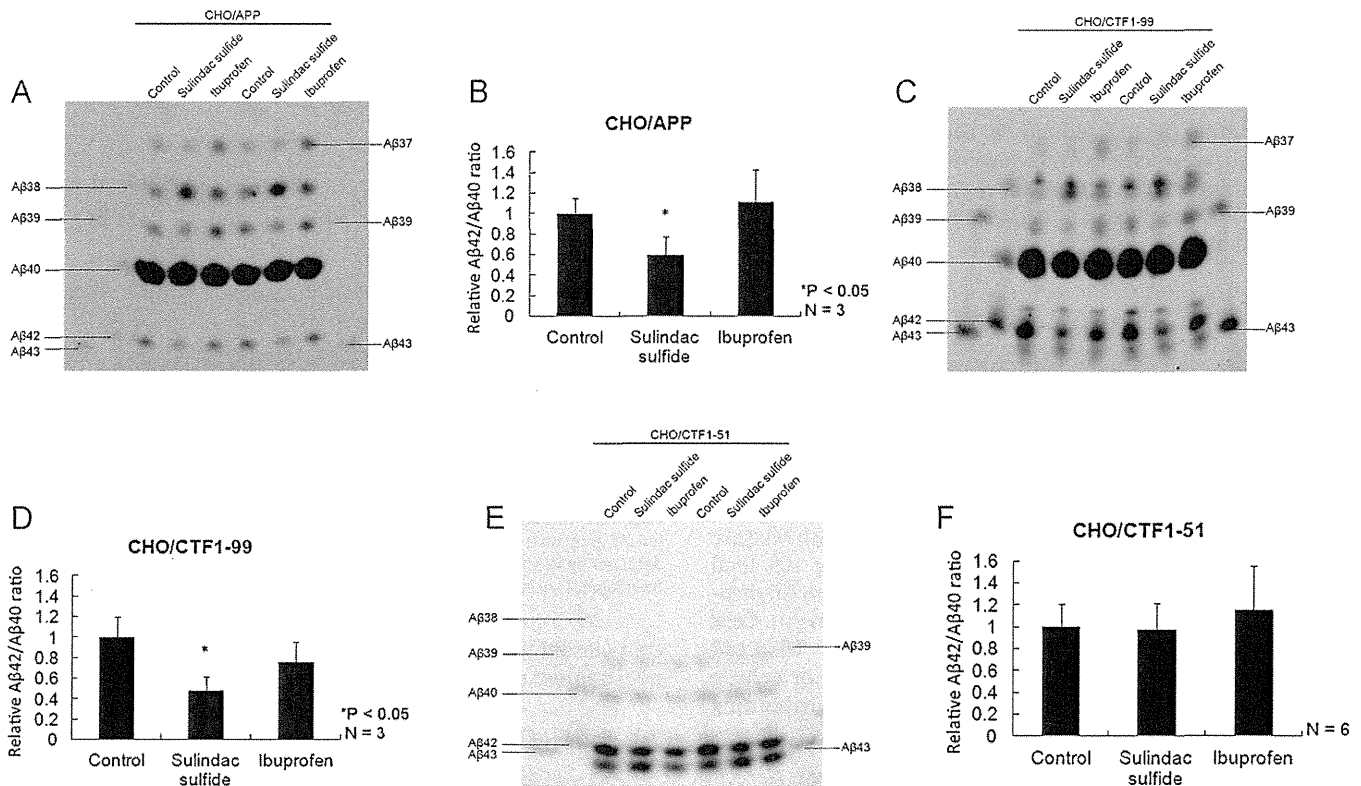


Fig. 2. Influence of GSMs on the A β 42/A β 40 ratio in stably-transfected CHO cells expressing APP (A and B), CTF1-99 (C and D), or CTF1-51 (E and F). (A, C, and E) CHO cells were incubated with 60 μ M sulindac sulfide or 250 μ M ibuprofen. After incubation for 24 h, the medium was concentrated and A β 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 A β 42/A β 40 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 A β at a higher A β 42/A β 40 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 A β region of the A β 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 A β 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 A β 42 to total A β . Thus, it is thought that CTF1-51 increases the A β 42/A β 40 ratio and may reduce the effects of A β 42-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 A β 42/A β 40 ratio change the conformation of presenilin-1, reducing the distance between the N- and C-termini [1]. Also, APP mutations increasing the A β 42/A β 40 ratio reduce the distance between the N- and C-termini of presenilin-1 [4], while APP mutations that reduce the A β 42/A β 40 ratio increase this distance [15]. Therefore, it is thought that production of A β 42 is influenced by the conformation of presenilin, especially the distance between the N- and C-termini. Since CTF1-51 is a precursor peptide of A β that yields a high ratio of A β 42 to total A β , it is thought that CTF1-51 may reduce the distance between the presenilin N- and C-termini. Other studies showed that A β 42-lowering GSMs increase the distance between the N- and C-termini of presenilin, and that an A β 42-raising GSM reduced

this distance [9,16]. Accordingly, it is thought that GSMs alter the production of A β 42 by changing the conformation of presenilin and that CTF1-51 may induce a conformational state that results in production of a high ratio of A β 42 and thus reduces the effect of A β 42-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 A β 42 and increase A β 38 levels [10]. Thus, CTF1-51 perhaps yields a high ratio of A β 42 because of the high dimerization capacity of its transmembrane sequence. Other studies showed that A β 42-lowering GSMs reduce the dimerization capacity of transmembrane sequences of an A β precursor peptide [13]. Accordingly, it is thought that GSMs alter the production of A β 42 by changing the dimerization capacity of the transmembrane sequences of A β 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 A β 42-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 A β 42, treatment of AD patients with GSMs under the condition that A β is produced at a high A β 42/A β 40 ratio in the brain may be unfavorable. To evaluate the efficacy of GSMs, studies on GSMs in models producing a high ratio of A β 42 are important, and it will contribute to the development of new therapies for AD based on the amyloid hypothesis.

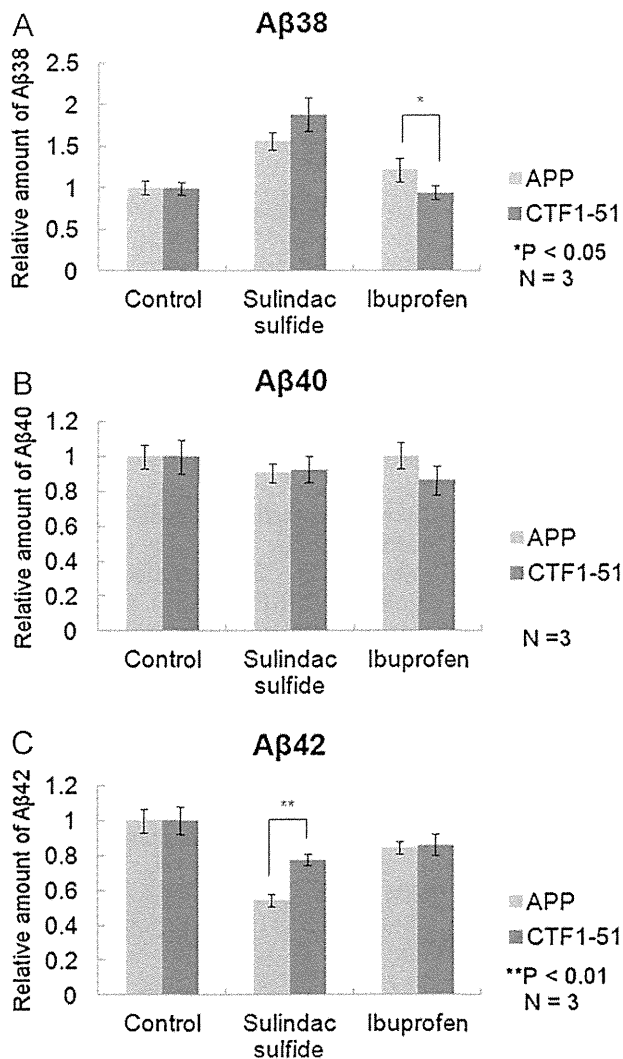


Fig. 3. Influence of GSMs on the production of A β 42, A β 40, and A β 38 by stably-transfected CHO cells expressing APP or CTF1-51. CHO cells were incubated with 60 μ M sulindac sulfide or 250 μ M ibuprofen. After incubation for 24 h, the medium was subjected to sandwich ELISA to measure A β levels. All samples were measured in duplicate or triplicate. Relative A β 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 A β 37/A β 40 ratio significantly increased, while A β 43/A β 40 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 A β 43 to A β 40 and then A β 40 to A β 37 in CHO cells expressing CTF1-99. This process from A β 43 to A β 37 should be confirmed in other systems in future.

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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 (A β) accumulation. It has been reported that A β 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 A β -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 A β accumulates but does not modulate the protease activity of NEP. © 2011 Wiley Periodicals, Inc.

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 (A β). A β 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 A β (Selkoe, 2002).

A β is produced from amyloid precursor protein (APP) by the action of β - and γ -secretases, although APP is usually cleaved within the A β sequence by α -secretase. A β is degraded by neprilysin (NEP; Iwata et al., 2001). NEP is a type II membrane metallopeptidase that is capable of degrading not only monomeric A β but also pathological oligomeric A β (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), A β generation occurs in lipid rafts (Wada et al., 2003). It has been reported that A β 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

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that cholesterol regulates the localization of mature NEP in lipid rafts, where the substrate A β 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 \times 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 \times FLAG vector, yielding FLAG-NEP E584V and FLAG-NEP E403C, respectively.

Antibodies

The following antibodies were purchased: anti-FLAG M2 (Sigma, St. Louis, MO); anti-flotillin-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% CO₂ 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 μ g/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 μ g/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 μ g 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 μ g/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 μ g/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 (M β CD; Trappsol) for 20 min in a CO₂ incubator at 37°C, and collected. Lipid rafts fractions were treated with 50 mM M β CD on ice for 1 hr, dissolved in a double volume of TBS containing Complete, EDTA-free protease inhibitors and 0.7 μ g/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 NaN₃. 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 μ M Z-Leu-Leu-Leu-H, and as a substrate 50 μ M Z-Ala-Ala-Leu-*p*-nitroanilide (ZALL-*p*-NA; Peptide Institute), in the presence or absence of 10 μ M 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

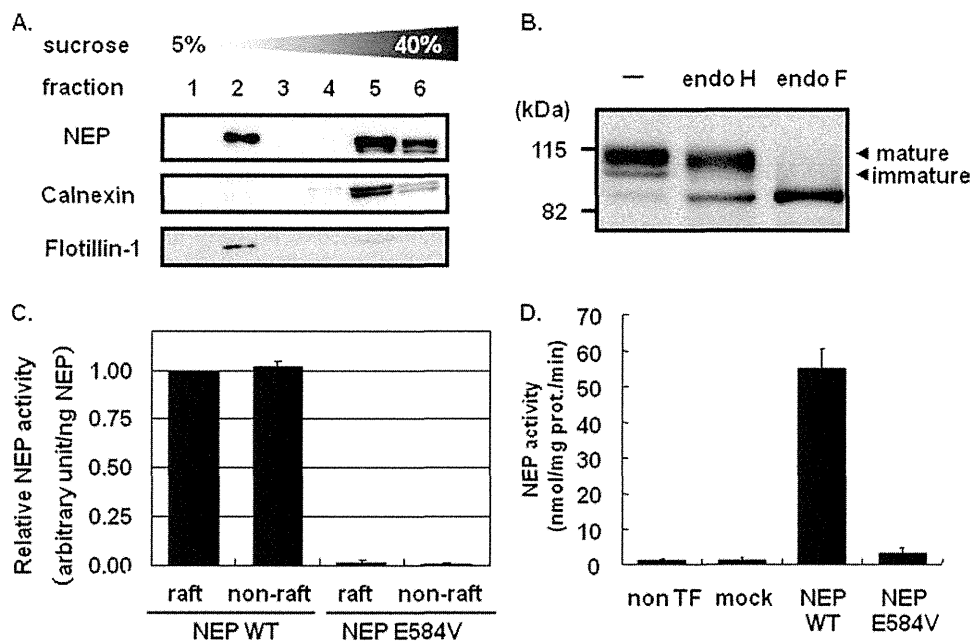


Fig. 1. NEP localization and activity in lipid rafts. **A:** Western blot analysis of lipid rafts fractionated from HEK293 cells overexpressing FLAG-NEP WT by a sucrose density-gradient centrifugation method. An anti-FLAG antibody was used to detect NEP. Lipid rafts were detected using an antibody raised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

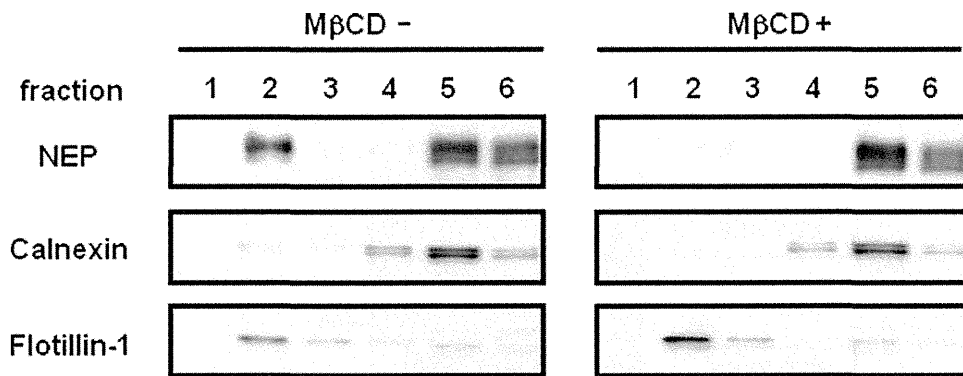


Fig. 2. Delocalization of NEP from lipid rafts in cells treated with M β CD. HEK293 cells overexpressing FLAG-NEP WT were treated with methyl- β -cyclodextrin (M β CD; +) or left untreated (-), and lipid rafts were fractionated as described in Materials and Methods.

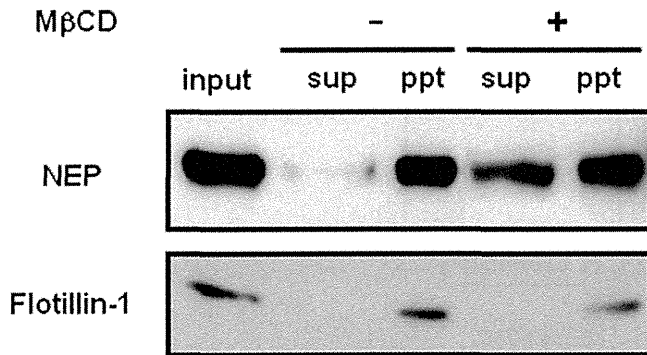


Fig. 3. Delocalization of NEP from fractionated lipid rafts after M β CD treatment. The lipid raft fraction, isolated from HEK293 cells overexpressing FLAG-NEP WT, was treated with (+) M β CD or left untreated (-) and separated into a supernatant (Sup) and a pellet (Ppt) by ultracentrifugation. The distribution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

WT of cholesterol by treating them with 10 mM methyl- β -cyclodextrin (M β CD) 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 M β CD 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 M β CD 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 M β CD. However, some of the NEP associated with lipid rafts was detected in supernatants prepared from lipid rafts treated with M β CD treatment. Flotillin-1 remained exclusively in the pellets, suggesting that flotillin-1 was not associated with cholesterol.

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.