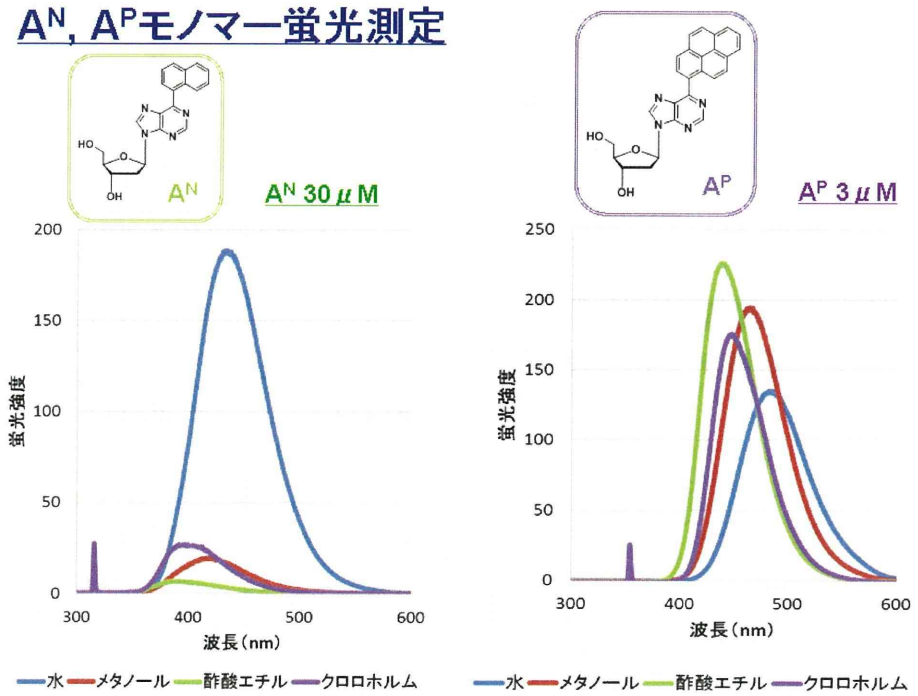
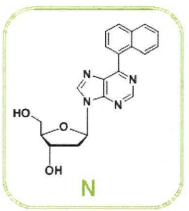
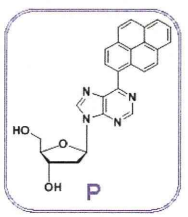
A^N, A^Pモノマー蛍光測定



オリゴ合成(1)

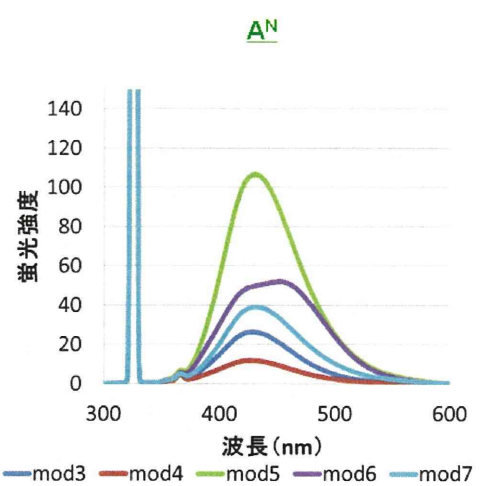
MALDI – TOF / MS 結果

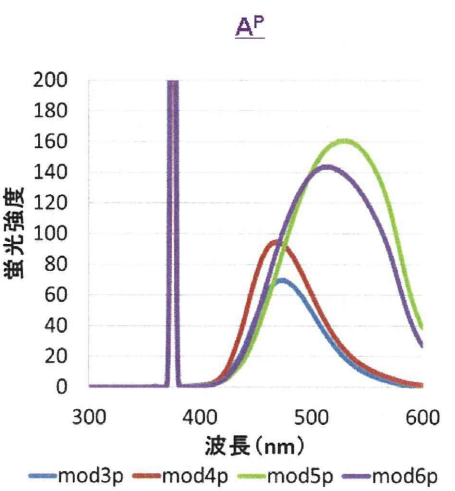




Name	sequence	calculated	observed
MOD1	5'-CGGCACGAGCGGC-3'	3985.59	3983.70
MOD2	3'-GCCGTGCTCGCCG-5'	3927.54	3925.88
MOD3	5'-CGGCA**N**CGAGCGGC-3'	4409.95	4411.06
MOD3p	5'-CGGCA**P**CGAGCGGC-3'	4484.03	4484.42
MOD4	3'-GCCGT**N**GCTCGCCG-5'	4351.89	4353.55
MOD4p	3'-GCCGT**P**GCTCGCCG-5'	4425.97	4426.70
MOD5	5'-CGGCA**N N**CGAGCGGC-3'	4834.30	4839.67
MOD5p	5'-CGGCA**P P**CGAGCGGC-3'	4982.46	4983.44
MOD6	3'-GCCGT**N N**GCTCGCCG-5'	4776.25	4780.25
MOD6p	3'-GCCGT**P P**GCTCGCCG-5'	4924.41	4929.29
MOD7	5'-CGGCA**N N N**CGAGCGGC-3'	5258.65	5261.49
MOD8	5'-CGGCA**A**CGAGCGGC-3'	4298.80	4297.08
MOD9	3'-GCCGT**A**GCTCGCCG-5'	4240.75	4241.29
MOD10	5'-CGGCA**AA**CGAGCGGC-3'	4612.01	4612.46
MOD11	3'-GCCGT**AA**GCTCGCCG-5'	4553.96	4549.67
MOD12	5'-CGGCA**AAA**CGAGCGGC-3'	4295.22	4296.76

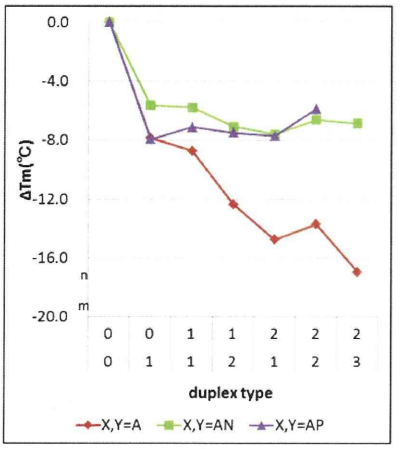
DNA一本鎖蛍光測定





オリゴマーTm測定

		X	Y	T_m (℃)
5'-CGGCA CGAGCGGC-3'	mon1			70.3
3'-GCCGT GCTCGCCG-5'				
5'-CGGCAXCGAGCGGC-3'	mon8	A	/	62.5
3'-GCCGT GCTCGCCG-5'	mon2	A^N	/	64.7
	mon2p	A^P	/	62.4
5'-CGGCAXCGAGCGGC-3'	mon9	A	A	61.6
3'-GCCGTYGCTCGCCG-5'	mon3	A^N	A^N	64.6
	mon3p	A^P	A^P	63.2
5'-CGGCAXXCGAGCGGC-3'	mon10	A	A	58.0
3'-GCCGT Y GCTCGCCG-5'	mon4	A^N	A^N	63.3
	mon4p	A^P	A^P	62.8
5'-CGGCA X CGAGCGGC-3'	mon11	A	A	55.6
3'-GCCGTYYGCTCGCCG-5'	mon5	A^N	A^N	62.7
	mon5p	A^P	A^P	62.6
5'-CGGCAXXCGAGCGGC-3'	mon12	A	A	56.6
3'-GCCGTYYGCTCGCCG-5'	mon6	A^N	A^N	63.7
	mon6p	A^P	A^P	64.5
5'-CGGCAXXXCGAGCGGC-3'	mon13	A	A	53.4
3'-GCCGT YY GCTCGCCG-5'	mon7	A^N	A^N	63.5

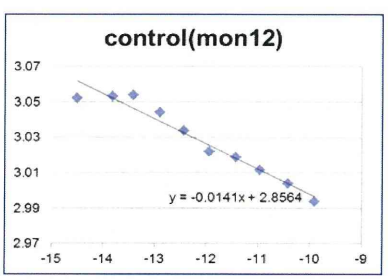


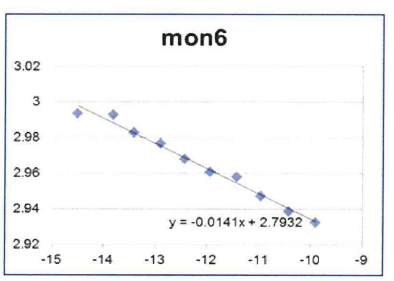
T_m were measured in a buffer of 10mM NaPhosphate (pH 7.0) and 100mM NaCl with 3 μ M of each oligomer.

オリゴマー van't Hoff 測定

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

$$1 / T_m = (R / \Delta H^\circ) / \ln(Ct/4) + (\Delta S^\circ / \Delta H^\circ)$$





	Sequence	ΔH° (kcal/mol)	ΔS° (cal/K・mol)	ΔG°_{298} (kcal/mol)
mon12	5'-CGGCAAACGAGCGGC-3' 3'-GCCGTAAGCTCGCCG-5'	-141.4	-404.0	-21.0
mon6	5'-CGGCANNCGAGCGGC-3' 3'-GCCGTNNGCTCGCCG-5'	-141.4	-395.1	-23.7

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
石井 純 田中 勉 荻野 千秋 近藤 昭彦	第3章 「細胞内生体分子群の実測定量解析」 4. フローサイトメトリーと GFP レポーターによる G 蛋白質シグナルのシングルセル解析	(監修) 神原秀記 松永是 植田充美	シングルセル解析の最前線	CMC 出版	日本	2010	

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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シングルセル解析の最前線

第3章　細胞内生体分子群の実測定量解析

4　フローサイトメトリーとGFPレポーターによるG蛋白質シグナルのシングルセル解析

石井　純[1)]，田中　勉[2)]，荻野千秋[3)]，近藤昭彦[4)]

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2)　神戸大学　自然科学系先端融合研究環　重点研究部　助教
3)　神戸大学大学院　工学研究科　応用化学専攻　准教授
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4　フローサイトメトリーとGFPレポーターによるG蛋白質シグナルのシングルセル解析

石井　純[*1]，田中　勉[*2]，
荻野千秋[*3]，近藤昭彦[*4]

4.1　はじめに

シングルセル解析（1細胞解析）とはその名のとおり，1細胞を解析する技術のことであるが，いったい何のメリットがあるのであろうか？

本節では筆者らがフローサイトメーターとGFPを利用して行った研究例をもとに，シングルセル解析の1つの利点と魅力を紹介する。

4.2　緑色蛍光蛋白質（GFP）とフローサイトメーター

オワンクラゲ*Aequorea victoria*由来の緑色蛍光蛋白質（Green fluorescent protein, GFP）は約27 kDaの分子量からなる蛋白質であり，青色の励起光をあてることにより緑色の蛍光を発する。GFPは単独で発色団を形成し，発色に基質を必要としないため，細胞内で発現させたGFPは細胞を非破壊の状態，かつリアルタイムでその蛍光を観察できる。そのため，GFPはレポーター遺伝子として広く普及し，その発見者である下村脩氏は2008年にノーベル化学賞を受賞した。分子生物学を含む生物系の研究者ならほとんど誰もが一度は耳にしている有名な蛋白質であろう。

フローサイトメトリーとは，微細な粒子を流体中に流し込み，整然と列をなして流れている個々の粒子にレーザー光をあてて反射する光を測定し，その光の強さを電気信号に置き換えて定量化する分析手法のことである[1)]。フローサイトメトリーに用いられる分析装置のことをフローサイトメーターと呼ぶ。粒子として細胞を流すことも可能で，生物分野においても広く使用されている。シース液を一定の速度で流しておき，中央部からサンプル液を注入することで検体が1つ1つ離れた状態で整列して流れるため，その流れにレーザー光を照射することで1細胞ごとの散乱光や蛍光の情報を得ることができる（図1）。なお，個々の細胞の散乱光と蛍光は光電子増倍管（Photo-multiplier tube, PMT）によって高感度に検出される。毎秒数万個の細胞をリアルタイムに分析することができ，各種パラメーターを自動かつ客観的に高分解能で測定できるとい

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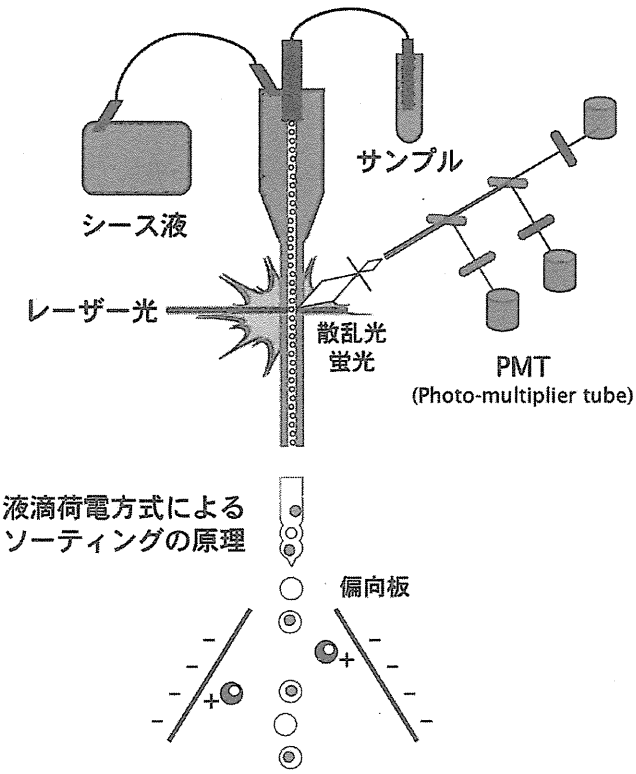


図１　フローサイトメーターのしくみ

う利点を持つ。目的の蛍光を発する細胞を分取することも可能で，分取機能を持つものをセルソーターと呼ぶ。近年のものでは，液滴荷電方式と呼ばれるソーティング（分取）形式を用いたタイプが多い。超音波発生装置による振動でレーザー照射部から少し離れた位置より１細胞ずつを含む液滴に分かれて落下させ，目的の蛍光などを発する細胞の液滴を荷電することで，偏向板により目的の細胞のみを分取できる（図１）。

フローサイトメーターは蛍光標識した抗体などを利用した抗原検出などに広く利用されているが，GFPをレポーターとする組み合わせにおいても威力を発揮する。筆者らの研究例をもとにその一例を見てみよう。

４.３　G蛋白質共役型受容体（GPCR）

G蛋白質共役型受容体（GPCR）は，７回膜貫通型の構造を有する表層蛋白質で細胞外リガンドが結合すると細胞内G蛋白質を介してシグナルを伝達する。GPCRは嗅覚，味覚，視覚あるいは心拍，血圧，神経伝達，細胞増殖など多様な生理機能に関与しており，その調節因子は米食品医薬品局（FDA）の承認医薬品の中でも約30％を占める最主要な創薬ターゲットとなっている[2)]。GPCRはヒトにおいて700～1,000種類程度存在していると見積もられており，リガンド未知のオ

ーファン受容体もまだ多く残されている。これらオーファン受容体を含めたGPCRは，創薬候補となりうる新規リガンドの探索や疾患に関与する作用機序の解明など様々な研究が行われている。

GPCRのアッセイには哺乳動物細胞が使われるのが一般的であるが，筆者らは酵母細胞を宿主とした異種GPCRアッセイ系の開発に取り組んでいる。酵母2ハイブリッド法（Y2H）に代表されるように，酵母細胞は膨大な遺伝子ライブラリを簡便にスクリーニングできるため，ヒトを含む異種GPCRを酵母で発現・アッセイできれば，非常に魅力的なツールとなりうるためである。たとえば，リガンド候補となるペプチドや抗体遺伝子などをライブラリとすることにより，ペプチド創薬あるいは抗体医薬として利用できる分子が取得できるかもしれない。あるいは，GPCRに部位特異的変異かランダム変異を導入したライブラリからスクリーニングを行うことにより大規模な構造解析にも利用できるであろう。酵母は最もシンプルな真核単細胞であり，多様なGPCRおよびシグナル伝達機構を有する高等真核生物に比べて非常にシンプルなGPCRシグナル伝達機構を持つため，アッセイ系を構築するのに非常に適している。実際，ヒトを含む異種GPCRが酵母細胞で機能的に発現し，酵母内G蛋白質を介してフェロモンシグナル伝達経路を活性化することが知られており，アッセイ系の構築に利用されている[3]。

4.4　酵母フェロモンシグナル伝達経路を利用したGFPレポーターによるGPCRアッセイ系

フェロモンシグナル伝達経路は酵母の接合（Mating）機構を誘導する際に必要であることが知られている[4]。酵母a型細胞において，内在性GPCR（Ste2）はリガンドであるフェロモン（α-factor）が結合することにより構造変化を起こし，細胞内の三量体G蛋白質（Gpa1/Ste4/Ste18）を不活性型から活性型（GDP結合型→GTP結合型）に変化させる。活性化されたG蛋白質はGpa1とSte4/Ste18複合体に解離することでシグナルを伝達する（図2）。活性化されたシグナルはMAPキナーゼと呼ばれるカスケードによって増幅され，Far1とSte12という蛋白質をリン酸化する。サイクリン依存性キナーゼ阻害剤としての機能を持つFar1蛋白質はリン酸化されることによりG1期で細胞周期を抑制し，接合準備を整えるために細胞周期を同調する。また，Ste12は転写因子をコードしており，リン酸化されることにより核内に移行しては接合に必要な種々の遺伝子の転写発現を誘導することも知られている。

筆者らはフェロモンシグナルにより転写誘導される*FUS1*という遺伝子に*GFP*遺伝子を融合することで，シグナル伝達に応答して蛍光が発現するシステムを構築した（図2）[5]。Y2Hでも利用されている*lacZ*のような比色による酵素反応用の遺伝子や*HIS3*のような生育セレクション用の遺伝子などによるGPCRアッセイ系はこれまでに開発されていたが，*GFP*をレポーター遺伝子として利用することによりセルソーターによる高速スクリーニングが可能となると考えたので

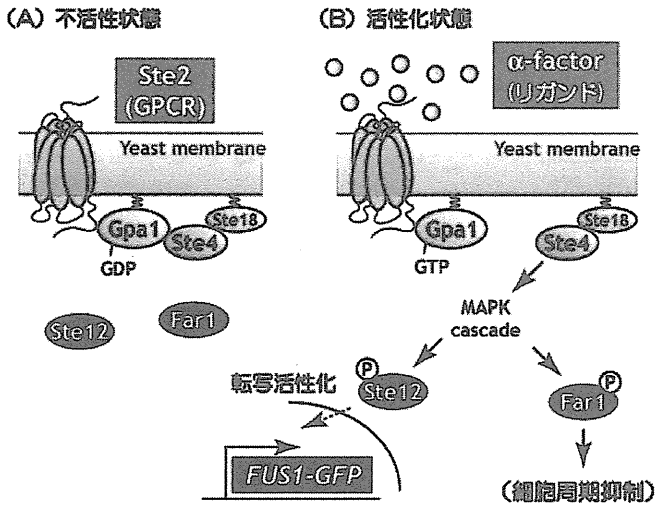


図２　酵母フェロモンシグナル経路を利用したGPCRアッセイ系

ある。酵母を利用したこれまでのシステムでは，細胞周期抑制を誘導するFar1をコードする遺伝子を破壊した株あるいは破壊していない株の両方が利用されており，*GFP*をレポーター遺伝子とした場合，どちらが適しているかを確認する必要があった。そこで，*GFP*レポーター遺伝子を組み込んだ株をもとに*FAR1*遺伝子を破壊した株を構築し，α-factorを添加することでシグナル伝達を引き起こし，*GFP*レポーター発現にどのような影響を与えるかを調べることにした。

4.5　*FAR1*遺伝子破壊株におけるシグナル伝達のフローサイトメトリー解析

*GFP*レポーター遺伝子を組み込んだ細胞にα-factorを添加した場合と添加していない場合でそれぞれ培養し，経時的に細胞をサンプリングしてフローサイトメーターで解析した。解析した細胞の緑色蛍光強度の平均値（１万細胞の平均値）を図３(A)に示す（白色棒グラフ：α-factorを添加していない細胞，黒色棒グラフ：α-factorを添加した細胞）。各細胞においてα-factorを添加することによってシグナルが伝達されてGFPが発現していることが確認され，どの細胞においても12時間後が最も蛍光強度が高くなることが分かった。また，*FAR1*遺伝子を破壊した株が相対的に高い蛍光強度を示すことも明らかとなった。このグラフはシグナル伝達量の平均値を反映したものであり，*GFP*の代わりに*lacZ*などの酵素をレポーター遺伝子として使用した場合でも，よく似た結果が得られると予想される。

一方で，フローサイトメーターで解析した結果の表示形式を変え，緑色蛍光を横軸とし，細胞数を縦軸としたヒストグラムで１万細胞をプロットした結果を図３(B)に示す（色付ヒストグラム：α-factorを添加していない細胞，白抜ヒストグラム：α-factorを添加した細胞）。結果は

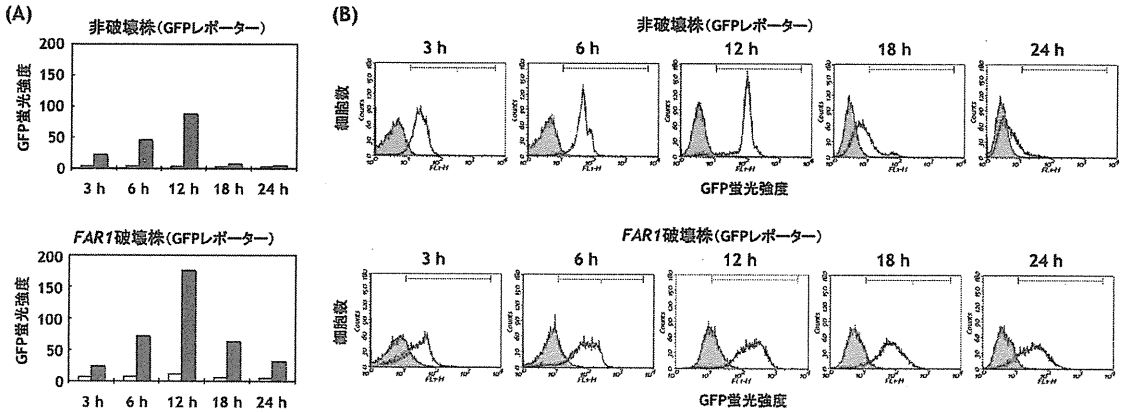


図3　*FAR1*破壊株におけるGFPレポーター発現のフローサイトメトリー解析

上述のグラフと同じものであるが，より細かい情報が見て取れる。α-factorを添加した細胞の12時間のプロットをそれぞれ見ていただくと，*FAR1*を破壊した細胞とそうでない細胞でヒストグラムの形状が明らかに違うことに気づくであろう。たとえば，上段の非破壊株ではGFPの発現量が非常に揃っていることが分かる。これは，*FAR1*非破壊株がシグナル伝達によって細胞周期抑制されて，細胞周期が一致していることを表していると考えられる。逆に下段の*FAR1*破壊株では，細胞周期が抑制されないため，細胞間でのGFPの蛍光強度（発現量）がばらついて一様に分布していると考えられる。このように，１細胞ごとの蛍光強度とその分布を付加的な情報として得ることができることはフローサイトメーターの分解能の高さを物語っており，シングルセル解析ならではの利点と言えるであろう。しかしながら，これらの結果では蛍光強度の高い*FAR1*破壊株を使うべきか，レポーター発現量のばらつきが少ない非破壊株を使用するべきかは人によって判断の異なるところであろう。

4.6　モデルGPCR発現系でのシグナル伝達のフローサイトメトリー解析

次に，異種GPCRを発現することを想定して酵母内在性GPCRが競合発現しないようにするため，Ste2をコードする遺伝子をノックアウトした。また，変異ライブラリなどに応用することも考え，GPCRの発現には環状エピソーム型（ゲノムに組み込まれず複製・保持される）プラスミドを用いることにし，今回はモデルとして異種GPCRの代わりに*STE2*遺伝子を発現させることとした。

図４にリガンドであるα-factorを添加してから，経時的に細胞の緑色蛍光をフローサイトメーターで解析した結果を示す。ゲノム上の*STE2*遺伝子を破壊していない細胞ではほぼ100％の細胞がGFP由来の蛍光を発していた（図４(A)）。しかしながら，ゲノム上の*STE2*遺伝子を破壊し，代わりにエピソーム型プラスミドでSte2を発現する細胞では，時間とともに蛍光を発してい

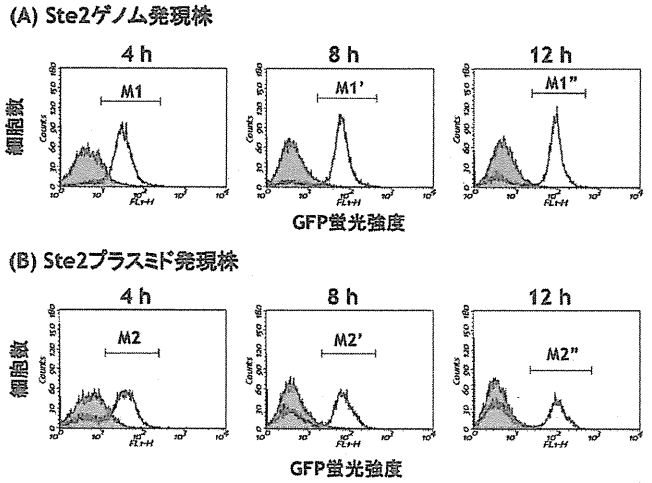
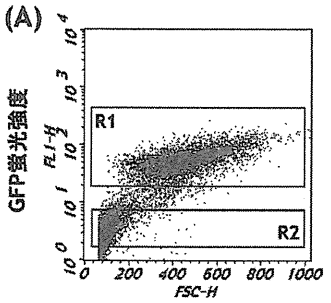


図４　エピソーム型プラスミドによるGPCR発現系におけるシグナル伝達のフローサイトメトリー解析

る細胞の割合が減少し，12時間後にはその割合はおよそ40％程度にまで落ち込んでいることが明らかとなった（図４(B)）。この結果も１細胞ごとの蛍光強度を解析できるフローサイトメーターの大きな利点を表していると言える。たとえば，フローサイトメーター以外の蛍光分析機器を用いた場合，細胞数あたりの蛍光強度は測定できるが，蛍光強度の異なる細胞群の存在やその割合までは通常解析することができない。そのため，単に蛍光強度が弱いという情報しか得られず，蛍光強度の異なる２つの細胞群が存在するということは予想し難いであろう。GFP蛍光を発していない細胞群はシグナルを活性化していないであろうことが予想され，これら２つの細胞群に分かれた原因に*STE2*遺伝子をゲノムからエピソーム型プラスミドに乗せ変えたことが関与していることは明らかである。筆者らは，蛍光を発していない細胞群がSte2を発現していない細胞群であると考え，シグナル伝達に起因したG1期での細胞周期抑制がプラスミドを欠落させているのではないかと仮説を立てた。

４.７　セルソーターによるシグナル活性化細胞群と非活性化細胞群の分取および解析

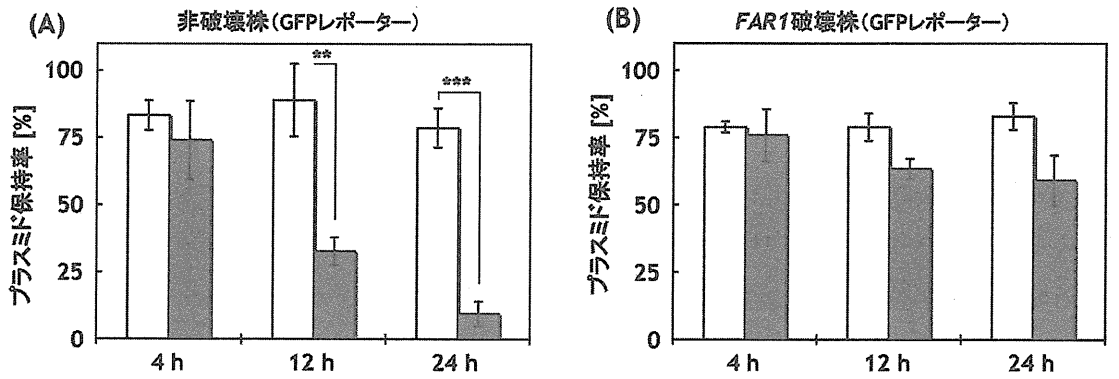
Ste2をエピソーム型プラスミドで発現させる細胞においてα-factor添加後12時間培養し，セルソーターを用いてシグナル活性化細胞群（GFP蛍光強度の高い細胞群）とシグナル非活性化細胞群（GFP蛍光強度の低い細胞群）を別々にソーティングして（図５(A)），それぞれのプラスミド保持率を調べた（図５(B)）。驚くべきことに，蛍光強度の高い細胞群（R1領域）のプラスミド保持率はほぼ100％であったのに対し，蛍光を発していない細胞群（R2領域）のプラスミド保持率はほぼ０％であった（図５(B)）。つまり，エピソーム発現型においてはプラスミドの欠落のためにシグナル伝達が起こらなくなり蛍光を失っていることが証明された。この結果は１細胞ご



(B)

プラスミド保持率	
R1 領域	R2 領域
97.4%	0.6%

図5　セルソーターによるシグナル活性化細胞群と非活性化細胞群のソーティングおよびプラスミド保持率の解析



(C)

時間 (h)	非破壊株	*FAR1*破壊株
4	88.3%	96.1%
12	37.2%	81.0%
24	11.6%	72.0%

図6　*FAR1*遺伝子破壊がプラスミド保持率へ与える効果

とに蛍光を解析できることに加え，目的細胞を分取できるセルソーターの大きな利点を証明している。また，筆者らの考えるGPCRアッセイ系でのセルソーターによる高速スクリーニングの実現可能性も示唆している。

これらフローサイトメーターを利用した解析結果から，シグナル伝達によるG1期での細胞周期抑制がプラスミドを欠落させているという仮説を確かめるため，*FAR1*破壊株と非破壊株にα-factorを添加後，経時的にサンプリングしてプラスミド保持率を調べた（図6(A)～(C)）。*FAR1*を破壊していない株では時間とともに明らかにプラスミド保持率が激減したのに対し，*FAR1*破壊株ではプラスミドの保持率が大幅に改善される結果となった（図6(C)）。このことから筆者らの仮説の妥当性が証明された。

遺伝子ライブラリからのスクリーニングを考えた場合，プラスミドが脱落するということはア

ッセイが不可能になる細胞が増えるというだけでなく，スクリーニングしたい遺伝子を回収できなくなるという致命的な問題につながることが予期されるため，*FAR1*遺伝子の破壊によってプラスミド保持率が維持されるという結果は蛍光レポーターを用いたGPCRアッセイ系によるハイスループットスクリーニング系を構築する上において有益な情報となるであろう。

*FAR1*遺伝子を破壊していない株がエピソーム型プラスミドを用いたGPCR発現系において，フローサイトメーターとGFPを用いなくてもレポーターの発現が悪くなることもしくはプラスミドの保持率に大きく影響することを予想できる研究者はいるかもしれない。しかしながら，筆者らが行ったようにシグナルを活性化している細胞群と活性化していない細胞群を分離して別々に解析することは，おそらくフローサイトメーターとGFPを用いた系以外ではほとんど不可能であったと自負しており，そこにシングルセル解析の利点と魅力が隠されていると思っている。

4.8　おわりに

フローサイトメーターおよびGFPが世に広く普及するようになってからもうすでに久しく年月を重ねており，これらの有用性は言うまでもない。フローサイトメーターとGFPの組み合わせによるシングルセル解析，ありきたりの組み合わせのように感じられるかもしれないが，まだまだ応用できる分野は広く存在すると思われる。筆者らは今回紹介した研究以外にもフローサイトメーターとGFPを利用して酵母用発現ベクターを簡便に評価できるシステムも報告しているので，もし興味のある方は御一読いただけると甚幸である[6)]。今回紹介したアプローチはあくまでシングルセル解析のほんの一例であり，なじみの薄いテーマであったかもしれないが，少しでもシングルセル解析の利点・魅力が伝わればと切に願っている。

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Research paper

Microtubule association of a neuronal RNA-binding protein HuD through its binding to the light chain of MAP1B

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ABSTRACT

RNA-binding proteins (RBPs) play a vital role in the post-transcriptional regulation of gene expression during neuronal differentiation and synaptic plasticity. One such RBP family, the neuronal Hu protein family, serves as an early marker of neuronal differentiation and targets several mRNAs containing adenine/uridine-rich elements. Recently, we reported that one of the neuronal Hu proteins, HuD stimulates cap-dependent translation through interactions with eIF4A and poly (A) tail. Nevertheless, little is known with respect to how neuronal Hu proteins contribute to the local translation of target mRNAs in neuronal differentiation. Here, we found that neuronal Hu proteins, but not the ubiquitously expressed HuR protein, directly interact with the light chain of microtubule-associated proteins MAP1B (LC1). We also show that HuD simultaneously binds both RNA and LC1 *in vitro* and that it tightly associates with microtubules in cells in an LC1-dependent manner, raising the possibility that HuD recruits target mRNAs to microtubules. These results uncover the neuronal binding partners for neuron-specific Hu proteins and suggest the involvement of Hu proteins in microtubule-mediated regulation of mRNA expression within neuronal processes.

1. Introduction

Accumulating evidence suggests that RNA-binding proteins (RBPs) play crucial roles in regulating the expression of various neuron-specific genes at the post-transcriptional level [1,2]. The presence of mRNAs and ribosomes in neuronal dendrites implies that the translation of these mRNAs is essential for neuronal differentiation and some forms of synaptic plasticity [3,4]. A recent study indicated that several RBPs, including neuronal Hu proteins, are specifically expressed in neurons in the mouse brain [5]. Furthermore, it now appears that neuronal Hu proteins are essential for proper neuronal development and plasticity [4,6–8].

Hu proteins were initially identified as autoimmune antigens in human paraneoplastic neurologic disorders, and are highly conserved RNA-binding proteins among vertebrates [9–12]. Hu proteins show extensive similarity with the *Drosophila* ELAV (embryonic lethal abnormal visual system) protein [9,13], which is required for the development and maintenance of the *Drosophila* nervous system, and is involved in post-transcriptional regulation of a neuronal genes [12,14–16]. There are four members of the Hu family in vertebrates, each being expressed from distinct genes [12]. HuB, HuC, and HuD are specifically expressed in neurons [9,11,17–22]. In contrast, HuR is ubiquitously expressed [11,23]. All four Hu proteins contain three RNA-binding domains (RBDs) of the RNP-consensus sequence and a linker region separating the two N-terminal RBDs (RBDs 1 and 2) from the C-terminal RBD (RBD 3) [11–13,24]. Although the RBDs are conserved among all of the Hu proteins, the linker regions are somewhat diverse. Previous studies

Abbreviations: MAP1A, microtubule-associated protein 1A; MAP1B, microtubule-associated protein 1B; LC1, microtubule-associated protein 1B Light Chain; LC2, microtubule-associated protein 1B light chain; mRNP, messenger ribonucleoproteins.

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revealed that mammalian Hu proteins bind specifically to AU-rich elements (AREs) in the 3′ untranslated regions of various mRNAs [25]. RBD1 and RBD2 are responsible for the binding to AREs [22,26], whereas RBD3 contributes to poly(A)-binding of Hu proteins [22,27]. Currently Hu proteins are thought to recognize specific ARE-containing target mRNAs by binding to both AREs and to the poly(A) tail. Hu protein binding is thought to regulate the translation and/or stability of the bound mRNAs. Indeed, several putative target mRNAs of Hu proteins were reported to play crucial roles in cell differentiation and proliferation [26,28–37]. We have previously shown that HuD enhances cap-dependent translation in a eIF4A- and poly(A)-dependent manner [38]. We have also shown that the HuD needs to interact with both eIF4A and the poly(A) tail to induce neurite outgrowth in PC12 cells [38]. However, the mechanism of how the neuron-specific Hu proteins function in neuronal process extension pathway remains unclear.

In this study, we set out to find the neuronal binding partners for HuD by yeast two-hybrid screening. We show here that the light chain of microtubule-associated protein MAP1B (LC1) interacts with neuronal Hu proteins. Additional *in vitro* and *in vivo* analyses show that neuron-specific Hu proteins bind the light chains of both MAP1A and MAP1B, and form a ternary complex of LC1-HuD-RNA. Moreover, HuD associates with the microtubules network in a LC1-dependent manner. These findings provide novel insight into how neuron-specific Hu proteins associate with microtubules and their roles in neurons.

2. Materials and methods

2.1. Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using the MATCHMAKER GAL4 Two-Hybrid System 2 (Clontech). A cDNA fragment encoding a mutant mouse HuD (HuDmt), which was described previously [39], was PCR-amplified using appropriate synthetic primers, introduced into the two-hybrid vector, pGBKT7, and transformed into yeast strain AH109. A mouse brain cDNA library (Clontech) was used for screening of proteins interacting with the mutant HuD.

2.2. Cell culture and transfection

PC12 and HeLa cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (For HeLa cells) and 5% horse serum (for PC12 cells).

PC12 cells were transiently transfected with 8 μg of plasmid DNA and 10 μl of Lipofectamine 2000 (Invitrogen) per 6 cm dish. HeLa cell were transiently transfected with 1.5 μg of plasmid DNA and 6 μl of PolyFect Transfection Reagent (QIAGEN) per 3.5 cm dish. The cells were harvested for biochemical analyses 24 h after transfection.

2.3. Plasmid construction and preparation of recombinant proteins

The plasmids encoding T7-tagged Hu proteins were described previously [40]. The plasmids encoding GST-Hu proteins were made by introducing the same cDNA fragments as in T7-tagged Hu plasmids followed by the segment encoding six histidine residues into the expression vector pGEX3X (GE). The plasmids encoding FLAG-tagged mouse LC1 and LC2 were made by introducing cDNA fragments corresponding to the carboxy-terminal 250 amino acids of MAP1B and the carboxy-terminal 222 amino acids of MAP1A into pFLAG-CMV (SIGMA), respectively. For the plasmids encoding myc-tagged LC1 and LC2 and the plasmids encoding MBP-LC1 and MBP-LC2, the same cDNA fragments as in FLAG-tagged LC plasmids were introduced into pCDNA3.1 (Invitrogen) and pMAL-c2 (New England BioLabs), respectively. Plasmids encoding GST- and MBP-fusion proteins were transformed into *Escherichia coli* XL2. GST- and MBP-fusion proteins were induced with 1 mM IPTG for 3 h and affinity-purified with glutathione-Sepharose 4B (GE) and nickel-agarose (QIAGEN), respectively.

2.4. In vitro binding experiment

GST pull-down assay was performed as described previously [39] and the bound proteins were separated by SDS-PAGE and immunoblotted with anti-myc monoclonal antibody (Roche) or anti-MBP antibody (GE). Poly(U) pull-down assay was performed as described previously [39] with a modification that the binding buffer contained 2 mg/ml heparin.

2.5. Immunological analyses

For immunoprecipitation analysis, HeLa cells transfected with FLAG-LC1 together with either T7-GFP, T7-HuD or T7-HuDmt were lysed in TNE buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and immunoprecipitated with anti-T7 monoclonal antibody (Novagen). The precipitated proteins were separated by electrophoresis and immunoblotted with anti-FLAG monoclonal antibody (SIGMA). For indirect immunofluorescence analysis to detect endogenous Hu proteins and LC1, NGF-differentiated PC12 cells were fixed with 4% formaldehyde and then incubated with HuD specific 16C12 antibody and anti-LC1 monoclonal antibody (Sigma), respectively. Subsequently, the cells were incubated with FITC-labelled anti-mouse IgG secondary antibody (DAKO) and analyzed using a fluorescence microscope (Olympus BX51). Detection of detergent-insoluble T7-HuD and FLAG-LC1 was performed according to the method [38] with some modifications: transfected cells were treated with 0.2% Triton X-100 for 1 min at room temperature, washed two times with PBS containing 10 μg/ml RNase A, 1 unit/ml RNase I for 5 min at room temperature, and were incubated with anti-T7 antibody and anti-FLAG rabbit polyclonal antibody (Sigma). Subsequently, the cells were incubated with Alexa 488-labelled anti-mouse IgG and Alexa 546-labelled anti-rabbit IgG secondary antibodies (Molecular Probe) and analyzed using a confocal laser-scanning microscope (Zeiss LSM5 PASCAL).

3. Result and discussion

3.1. Identification of LC1 as a HuD-interacting protein

The yeast two-hybrid screening for the proteins that interact with RNA-binding proteins often result in many false positives, possibly because of indirect and non-specific RNA-bridged interactions by the RNA-binding activity of Hu proteins in cells. To circumvent such a problem, we used a mutant HuD protein that cannot bind RNA (HuDmt, [39]) as a bait for two-hybrid screening for the binding partners for HuD in neurons. After screening of 8×10^5 transformants from a mouse brain cDNA library, we obtained two positive clones that encode the carboxy-terminal 206 and 172 amino acids of MAP1B (Fig. 1A). MAP1B is a polyprotein that is expressed predominantly in the neuronal cells and is involved in microtubule stability [41]. The light chain of MAP1B (LC1), consisting of the carboxy-terminal part of 250 amino acids, is produced together with the heavy chain after proteolytic cleavage. Since the clones that we isolated corresponded to the majority of LC1, it was suggested that full-length LC1 also interacts with HuD.

To confirm whether this interaction occurs in mammalian cells, we first performed cotransfection with T7-tagged HuD and FLAG-tagged LC1 into HeLa cells. Immunoprecipitation using anti-T7

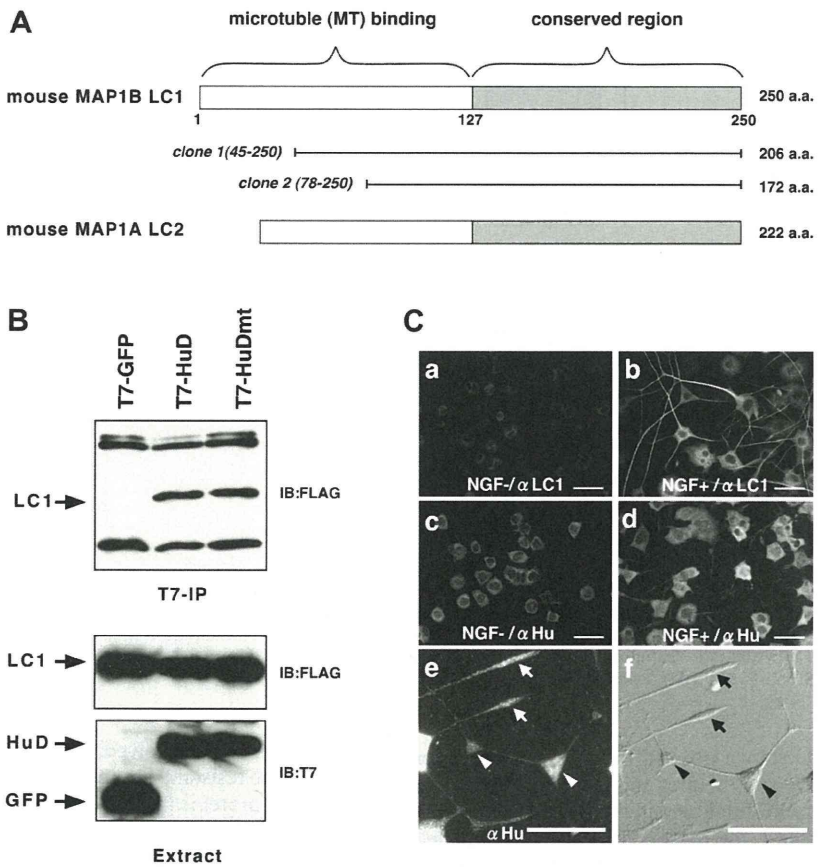

Fig. 1. Interaction between HuD and LC1 and their expression patterns in PC12 cells. A. Schematic representation of the light chains of MAP1B and MAP1A, LC1 and LC2. The LC1 regions encoded by the two-hybrid clones (clone 1 and clone 2) isolated are indicated. B. Specific coimmunoprecipitation of LC1 with HuD. FLAG-tagged LC1 was cotransfected with either T7-tagged GFP, HuD or HuDmt into HeLa cells. Extracts from the transfected cells were immunoprecipitated with anti-T7 antibody and the precipitates were immunoblotted (IB) with anti-FLAG antibody (T7-IP). For quantitative controls, each extract was immunoblotted with either anti-FLAG antibody or anti-T7 antibody (Extract). C. Colocalization of endogenous LC1 with neuron-specific Hu proteins. Undifferentiated (NGF–, a, c) and differentiated (NGF+, b, d, e: 5 days after addition of NGF) PC12 cells were immunostained with anti-LC1 antibody (a, b) or 16C12 antibody (c, d, e). Nomarski view of panel (e) is shown (f). Arrows and arrowheads indicate apical and branching regions of neuronal processes, respectively. Scale bars, 10 μm.

antibody followed by western blotting with anti-FLAG antibody showed that both wild-type and mutant HuD interact with LC1 (Fig. 1B). We then performed immunoprecipitation with monoclonal antibodies for endogenous neuron-specific Hu proteins and LC1 to evaluate the relevance of the interaction in PC12 cells, but failed to detect any interaction. This is possibly due to the monoclonal antibody binding perturbing the interaction between HuD and LC1. Thus, we examined endogenous expression of LC1 and HuD in PC12 cells before and after differentiation by nerve growth factor (NGF) (Fig. 1C). Immunostaining with LC1-specific monoclonal antibody showed that endogenous LC1 increased greatly after NGF stimulation and localized predominantly within neurites, indicating its association with microtubules. Endogenous HuD proteins also increased after NGF stimulation and localized within both the cytoplasm of cell bodies and neurites. Within neurites, they accumulated at the branching and apical regions. The localization patterns of the HuD and LC1 were somewhat different, but both were present in neurites, suggesting that HuD interacts with LC1 in PC12 cells.

3.2. Direct binding of the light chains to neuron-specific Hu proteins

LC2, the light chain of another microtubule-associated protein MAP1A, shows 49% amino acid sequence identity to LC1, and in particular, the carboxy-half region is highly conserved between the two light chains (76% identity) [43]. Thus, to test whether the LC1 binding to HuD is reproducible *in vitro* and whether LC2 also binds HuD, we performed GST pull-down assays using purified recombinant GST-HuD and purified light chains fused with maltose-binding protein (MBP). GST pull-down assays followed by western blotting with anti-MBP antibody showed that the binding between HuD and LC1 or LC2 is specific and direct (Fig. 2A). Next we wished to identify the region in LC1 that mediates the HuD interaction. Since there is less homology between the N-terminal region of LC1 and LC2, we reasoned that the C-terminal highly conserved region of LCs is involved in the binding to HuD. To examine this possibility, we performed GST-pull down assays using N-terminal and C-terminal deletion LC1 proteins and found that C-terminal but not with N-terminal region of LC1 copurifies with GST-HuD. In sum, we conclude that the highly conserved C-terminal region of LC1 is responsible for HuD binding.

Next, we tested whether only neural Hu proteins are bound to LC1. To address this question, we performed GST pull-down assays using MBP-LC1 and GST fusions of HuB, HuC and HuR. The results showed that LC1 also binds two other neural Hu proteins (HuB and HuC), but not HuR (Fig. 3). LC2 also bound the neural Hu proteins specifically (not shown).

3.3. Formation of the ternary complex between HuD, LC and RNA

Since HuD binds RNAs with AU-rich element (ARE), we examined whether the light chain binding and the RNA-binding are

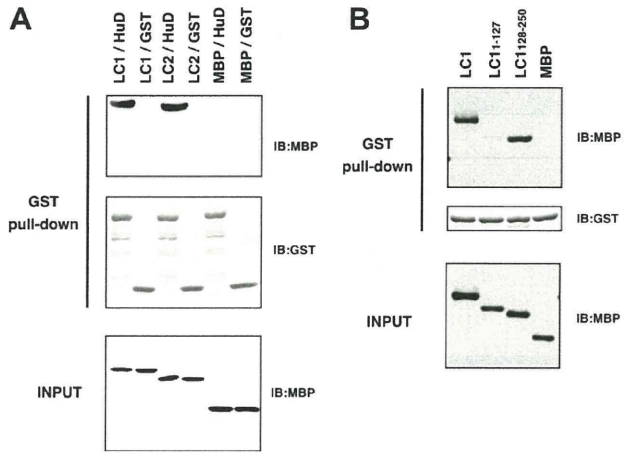

Fig. 2. Direct protein–protein interactions by HuD with LC1 and LC2. A. RNA-independent interactions between HuD and LCs. Purified MBP-LC1, MBP-LC2 and MBP were incubated with GST-HuD or GST proteins. GST pull-downs were examined for copurification of LCs by immunoblotting anti-MBP antibody (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (middle panel). Input MBP-LC1 and MBP proteins were shown in lower panel. B. C-terminal conserved region is required for LC1 to associate with HuD. Recombinant GST-HuD was incubated with the indicated MBP-LC1 proteins. GST-pull-downs were examined for copurification of LC1 proteins by immunoblotting with anti-MBP antibody (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (lower panel). Input LC1 proteins were shown below.

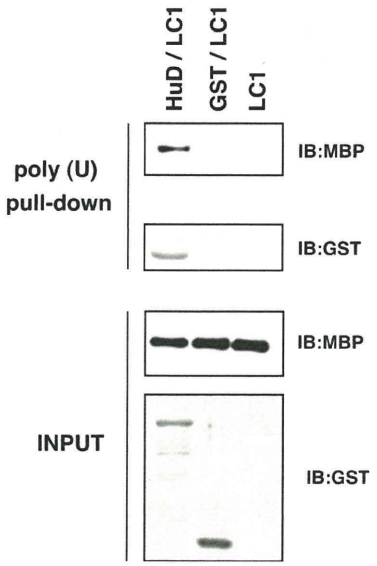

Fig. 4. Simultaneous binding of HuD to RNA and LC proteins *in vitro*. MBP-LC1 was incubated with GST-HuD or GST and pulled down with poly(U)-Sepharose beads, followed by immunoblotting with anti-MBP antibody. Lower panel showed that GST-HuD, but not GST, was pulled down with poly(U)-Sepharose beads. Input proteins were shown below.

compatible for HuD. To do so, we utilized the affinity of HuD for poly(U) RNA, which is carried by the first and second RNA-binding domains, representing its ARE-binding activity [44]. After mixing GST-HuD with either MBP-LC1, the reaction mixtures were pulled down with poly(U)-Sepharose beads followed by western blotting with anti-MBP antibody (Fig. 4). The results showed that LC1 were precipitated by poly(U)-Sepharose beads only in the presence of GST-HuD. This clearly showed that HuD can bind *in vitro* the light chains and RNA simultaneously, suggesting a similar ternary complex is formed in cells which is composed of HuD, LC and ARE-containing mRNA.

3.4. LC1-dependent association of HuD with microtubules

It was reported that LC1 associates with microtubules in the absence of its heavy chain when it was transiently expressed in non-neuronal cells [45,46]. In addition, it was suggested that HuB associates with microtubules in neuronal cells [47]. We then examined whether HuD also associates with microtubules and, if so, whether LC1 is involved in the association. To do so, we utilized a detergent-resistant property of microtubules with microtubule-associated proteins [45]. In this experiment, cells transfected with T7-tagged HuD with or without FLAG-tagged LC1 were mildly treated with a detergent before fixation and immunostaining with anti-T7 antibody (Fig. 5A). After the treatment, HuD remained in the cytoplasmic region only when it was coexpressed with LC1, whereas the control GFP disappeared even in the presence of LC1. LC1 remained filamentously in the cytoplasmic region, indicating its tight association with microtubules as described previously [45]. Moreover, the HuD co-localized precisely at the LC1 filaments after detergent treatment (Fig. 5B). On the basis of these results, we suggest that HuD associates with microtubules via its LC binding.

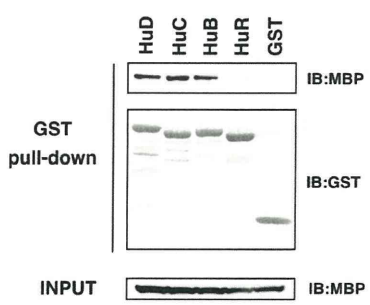

Fig. 3. LC1 specifically binds to neural Hu proteins. Purified MBP-LC1 was incubated with GST-HuB, GST-HuC, GST-HuD, or GST-HuR. GST pull-downs were examined for copurification of LC1 by immunoblotting with anti-MBP antibody (upper panel) and for pull-down efficiency by immunoblotting with anti-GST antibody (lower panel). Input LC1 proteins were shown below.

3.5. Possible roles of neuron-specific Hu proteins

Microtubules, whose dynamics are modulated by microtubule-associated proteins, play an important role in elaboration and maintenance of neuronal processes. Based on the timing of their expression, MAP1A and MAP1B are thought to regulate neuronal process extension during neuritogenesis and in mature neurons, respectively [48]. We have shown here that LC1 and LC2 are neuronal binding partners for the neuron-specific Hu proteins (Figs. 1 and 2). The specific binding of the light chains to neuron-specific Hu proteins parallels our previous finding that only the neuron-specific Hu proteins have an activity to induce neurite outgrowth when they are overexpressed in PC12 cells [40,49]. This suggests an important role of the light chains in the mechanism of the neurite induction by neuron-specific Hu proteins. In this connection, since functional analyses of LC1 revealed that it stabilizes microtubules and that the LC1 activity is inhibited in the presence of the MAP1B heavy chain, it was proposed that the heavy chain might act as the regulatory subunit of the MAP1B complex to control LC1 activity [50]. If this is the case, one possible role of neuron-specific Hu proteins may be to compete with the heavy chain inhibitory effect on LC1 and to promote microtubule stability in neuronal processes.

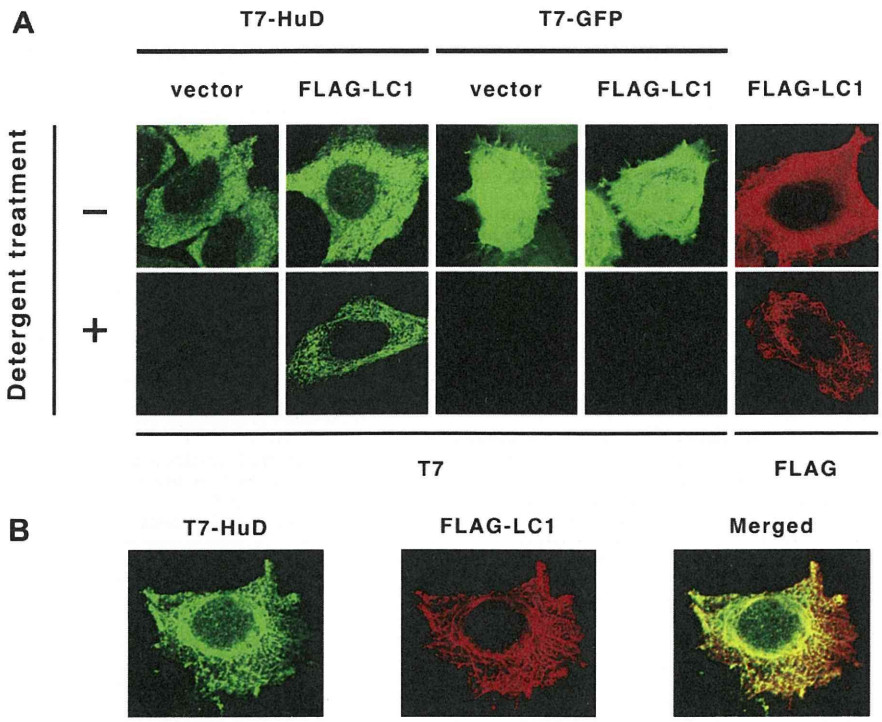

Fig. 5. LC1-dependent microtubule association of HuD. A. HeLa cells were transfected with T7- and FLAG-tagged proteins indicated above. Twenty-four hours after transfection, cells were treated with (+) or without (−) detergent solution, fixed, immunostained with anti-T7 antibody or anti-FLAG-antibody and analyzed by a confocal laser-scanning microscope. B. A HeLa cell expressing both T7-HuD and FLAG-LC1 was treated with detergent solution, fixed, immunostained with anti-T7 antibody (green) or anti-FLAG-antibody (red) and analyzed by a confocal laser-scanning microscope. Merged view is shown on the right (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Another possible and attractive role of neuron-specific Hu proteins is that they may be involved in microtubule-mediated regulation of transport and/or translation of some ARE-containing mRNAs, since HuD forms a ternary complex including one of the light chains and RNA *in vitro* (Fig. 4). This idea is consistent with LC1-dependent granular distribution of HuD on the LC1-associated microtubules in cells (Fig. 5). There is growing evidence that localization of mRNA and its regulated translation are important elements in determining cell polarity and identity in many organisms [51]. In particular, the microtubule network is known to serve as a major railway system for transport and localization of mRNA in neurons [52]. Thus, HuD may act as an adaptor for long-distance transport of some ARE-containing mRNAs along the neuronal processes. In fact, we previously demonstrated that HuD specifically interacts with TAP/NXF1, the primary mRNA nuclear export receptor and suggested that HuD is an adapter molecule for TAP/NXF1 involved in specific nuclear export and translational regulation in nerve cells [42]. On the other hand, Tretyakova et al. reported and proposed the model that LC1 tethers the mRNAs that form the mRNP (messenger ribonucleoproteins) complexes with TAP/NXF1 and several kinds of RNA binding proteins to microtubules [53]. Moreover, HuD and Tau mRNA were identified within same RNPs [54] and HuD was co-localized with GAP-43 mRNA [55]. Recently, we reported that HuD stimulates cap-dependent translation in a eIF4A- and poly(A)-dependent manner and this ability is prerequisite for neurite-inducing activity of HuD [38]. Taken together, we suggest that HuD may make a contribution to localized translation in neuron.

Finally, identification of mRNAs that associate with HuD-LC1 complex will provide important insights into the roles of Hu proteins in neuronal cells.

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