RNA interference (RNAi)を用いた変異型プリオン遺伝子特異的ノックダウン

分担研究者：北條　浩彦　国立精神・神経センター神経研究所

研究要旨 遺伝性プリオン病の原因遺伝子である変異型プリオン（*PrP*）遺伝子の発現を特異的に抑制する対立遺伝子（アリル）特異的RNAi誘導の実現を目指し、アリル特異的RNAi効果を簡便に評価するシステムを用いたsiRNAのスクリーニングを行った。レポーターアリルを利用した本システムを用いることで、従来では解析が不可能であったヘテロの状態での、変異型アリルに対するRNAiノックダウン効果と正常型アリルに対するRNAiの影響を同時に調べることができる。このスクリーニングによって変異型プリオン遺伝子を効果的にノックダウンするsiRNAを選定した。

A. 研究目的

ヒトプリオン遺伝子には多くの遺伝子変異が知られている。しかも、その多くは、アミノ酸変化を伴う塩基置換であり、さらに遺伝性のプリオン病との有意な関連も報告されている。この遺伝性プリオン病に関連する変異プリオン遺伝子の発現を特異的に抑制することができれば、遺伝性プリオン病の治療・予防に大きく貢献することができると考えられる。そこで今日、遺伝子機能阻害方法として幅広く利用されているRNA interference (RNAi)技術を用いて、遺伝子変異を持った対立遺伝子（変異対立遺伝子）だけを特異的にノックダウンする対立遺伝子（アリル）特異的RNAi誘導法の確立とその評価方法の確立を試みた。

アリル特異的RNAi誘導を実現させるためには、まず、その評価方法を確立しなければならない。昨年度において、レポーター対立遺伝子（レポーターアリル）を用いた簡便な評価システムの確立を行った。本年度は、そのシステムを用いて、変異型プリオン遺伝子に対する特異的なRNAiノックダウンを誘導するsiRNAのスクリーニングを実施し、高いノックダウン・ポテンシャルを持ったsiRNAを選定した。

B. 研究方法

ホタル・ルシフェラーゼ遺伝子とウミシイタケ・ルシフェラーゼ遺伝子をそれぞれコードした発現プラスミドを利用してレポーターアリルを構築した。まず、変異アリル、正常アリルに相当するオリゴDNAを合成し、それらをそれぞれのレポーター遺伝子の３'非翻訳領域に挿入して変異レポーターアリルそして正常レポーターアリルを構築した。

変異アリルをターゲットとする合成siRNAを作製し、正常、変異レポーターアリルをそれぞれ含んだプラスミドDNAとベーター・ガラクトシダーゼ遺伝子を含んだ発現プラスミドDNA（コントロールとして用いた）をリポフェクタミン２０００試薬（Invitrogen社）を用いたリポフェクションによってヒトHeLaまたはHEK293細胞に導入し、２４時間後、細胞抽出液を調製した。

得られた細胞抽出液を用いて、発現した両ルシフェラーゼ活性そしてコントロールのベーター・ガラクトシダーゼ活性を測定した。そして、ベーター・ガラクトシダーゼの活性値を基に両ルシフェラーゼの活性量（発現量）を正常化し、テストしたsiRNAの変異型アリルに対するノックダウン効果と正常型アリルに対する影響を評価した。

C. 研究結果

上記評価システムを用いて、プリオン遺伝子変異型アリルに対するアリル特異的ノックダウンを誘導するsiRNAをスクリーニングした。ターゲットとしたプリオン遺伝子変異型アリルは、Gerstmann-Straeussler syndromと関連するp102LとP105L変異、Creutzfeld-Jakob syndromと関連するD178N変異である。それぞれアミノ酸置換を伴う一塩基変異の対立遺伝子(アリル)である。これらの変異型アリルに対するsiRNAを設計・合成し、その効果を上記の簡易システムを用いて検討した。その結果、設計した一部のsiRNAは、変異型アリルと正常型アリルを識別し、変異型アリルに対する強いノックダウン効果を示すことが観察された。

我々は、さらにアリル特異的RNAiを増強させるために、変異型アリルと正常型アリルの識別を強めるsiRNAの設計を試みた。我々は、siRNA配列内にミスマッチを導入したsiRNAを設計し、そのアリル特異的RNAiノックダウン効果を検討した。その結果、一部のミスマッチ配列の導入は、アリル特異的ノックダウン効果を強めることが示唆された。

D. 考察

プリオン遺伝子の3つの変異型対立遺伝子（P102L, P105L, D178N）に対するsiRNAを設計し、アリル特異的RNAiノックダウン効果を調べた。その結果、それぞれのターゲット変異アリルによってノックダウン効果が異なっていた。ターゲットとなる変異型アリルの塩基置換の種類やその周りの配列によって、アリルの識別やノックダウンの効果が異なることが考えられる。

変異型アリルに対するsiRNAに、さらにミスマッチ塩基配列を導入することで、アリルの識別やアリル特異的ノックダウン効果が変化することが示された。アリル特異的ノックダウン効果を増強するミスマッチも観察されたことから、この様な、効果を持つミスマッチ配列の導入は有用であると考えられる。

本研究で用いたレポーター対立遺伝子を使ったRNAiの評価システムは、簡便かつ短時間で結果の得られるシステムであり、さらに、従来の方法では解析不可能であった正常型/変異型アリルがヘテロで存在する条件下でもそれぞれのアリルに対するRNAi効果を検討することができる。これによって、siRNAにミスマッチ配列を導入した場合の効果の検討を容易に行うことができた。このシステムを用いることで、siRNAの化学修飾によるアリル特異的ノックダウン効果も容易に検討できると考えられる。よって、どの様な化学修飾が有効であるかは、このシステムを使っての今後の検討課題である。

E. 結論

アリル特異的RNAiを誘導する場合、ターゲットとなる変異アリルによってその効果が異なる。ターゲットの変異型アリルがもつ塩基置換の種類やその周りの配列によって、アリルの識別・ノックダウン効果が異なること考えられる。本研究で用いたレポーター対立遺伝子を使ったアリル特異的RNAiの評価システムは、そのような違いを簡便に評価できるシステムであり、RNAiを用いた研究開発・応用に貢献できると考えられる。

F. 健康危険情報

とくになし。

G. 研究発表

1. 論文発表

1) Ohnishi Y, Tokunaga K, Kaneko K, Hohjoh H: Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles. J RNAi Gene silencing, 2: 154-160. 2006

2) Sakai T, Hohjoh H: Gene silencing analyses against amyloid precursor protein (APP) gene family by RNA interference. Cell Biol Int, 30:

952-956

3) Ohashi J, Naka I, Toyoda A, Takasu M, Tokunaga K, Ishida T, Sakaki Y, Hohjoh H: Estimation of the species-specific mutation rates of the DRB1 locus in human and chimpanzee. Tissue Antigens, 68: 427-431, 2006

4) Kawashima M, Tamiya G, Oka A, Hohjoh H, Juli T, Ebisawa T, Honda Y, Inoko H, Tokunaga K. Genome-wide association analysis of human narcolepsy and a new resistance gene. Am J Hum Genet, 79: 252-263, 2006

5) 北條浩彦: 神経・筋疾患治療への RNAi 応用. 神経治療学, 23: 31-36. 2006

6) 北條浩彦: RNAi 効果の評価法. バイオテクノロジージャーナル, 6: 51-57. 2006

2. 学会発表

1) Ohnishi Y. Yoshida M, Tamura Y, Tokunaga K, Kimura H, Hohjoh H: Assessment of allele-specific gene silencing by siRNA duplexes and short hairpin RNAs with wild and mutant-type reporter alleles. 56th Annual Meeting of the American Society of Human Genetics, New Orleans, Louisiana, USA, 2006

2) Ohnishi Y, Tokunaga K, Hohjoh H: Evaluation assay system for siRNA duplexes conferring allele-specific gene silencing. 20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, Kyoto, 2006

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

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
金子清俊	牛海綿状脳症と人変異型クロイツフェルト・ヤコブ病	本間清一	食品の安全性評価の考え方 -畜産食品を中心に-	日本栄養・食料学会	東京	2006	29-45
金子清俊	科学者が語るBSEのはなし	金子清俊	科学者が語るBSEのはなし	コープ出版	東京	2006	1-34
桑田一夫	２０世紀の２大発見－量子力学と分子生物学	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	9-24
桑田一夫	フーリエ変換とタンパク室・核酸の基本立体構造	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	25-49
桑田一夫	タンパク室・核酸の構造ダイナミクス	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	91-108
桑田一夫	計算機実験の基礎	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	137-145
桑田一夫	分子構造と生理機能	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	168-181
桑田一夫	タンパク質の構造異常	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	182-194
桑田一夫	タンパク質のコンホメーション制御－分子手術法	桑田一夫	『論理的創薬入門－構造生物学に基づくアプローチ』	共立出版	東京	2006.6	195-209

北條浩彦	巻頭ガイド 第２章 第６章 用語解説	程久美子 北條浩彦	RNAi実験なるほどＱ＆Ａ	羊土社	東京	2006	60-68 115-131
Hachiya NS, Kaneko K	Investigation of laser microdissected inclusion bodies	Berns M, Greulich K O	Method in Cell Biology	Academic Press	New York	2007	in press

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
Ohkubo T, Sakasegawa Y, Toda H, Kishida H, Arima K, Yamada M, Takahashi H, Mizusawa H, Hachiya NS, Kaneko K	Three-repeat tau 69 is a major tau isoform in laser-microdissected Pick bodies	Amyloid	13	1-5	2006
Kaneko K, Hachiya NS	The alternative role of 14-3-3 zeta as a sweeper of misfolded proteins in disease conditions	Med Hypotheses	67	169-171	2006
Furuya K, Kawahara N, Yamakawa Y, Kishida H, Hachiya NS, Nishijima M, Kirino T, Kaneko K	Intracerebroventricular delivery of dominant negative prion protein in a mouse model of iatrogenic Creutzfeldt-Jakob disease after dura graft transplantation	Neurosci Lett	402	222-226	2006
Noma T, Ikebukuro K, Sode K, Ohkubo T, Sakasegawa Y, Hachiya NS, KanekoK	Selection of DNA aptamers toward a specific protein in complex targets	Biotechnol Lett	28	1377-1381	2006
Ohnishi Y, Tokunaga K, Kaneko K, Hohjoh H	Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles	J RNAi Gene Silencing	2	154-160	2006
Kimura K, Moriwaki H, Nagaki M, Saio M, Nakamoto Y, Naito M, Kuwata K, Chisari FV	Pathogenic role of B cells in anti-CD40 caused necroinflammatory liver disease	Am J Pathol	168(3)	786-795	2006

Sukegawa HK, Kato Z, Nakamura H, Tomatsu S, Fukao T, Kuwata K, Orii T, Kondo N	Effect of Hunter disease (mucopolysaccharidosis type Ⅱ) mutations on molecular phenotypes of iduronate-2-sulfatase : Enzymatic activity, protein processing and structural analysis	J Inherit Metab Dis	29	755-761	2006
Ohnishi Y, Tokunaga K, Kaneko K, Hohjoh H.	Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles.	J. RNAi Gene silencing	2	154-160	2006
Sakai T, Hohjoh H	Gene silencing analyses against amyloid precursor protein (APP) gene family by RNA interference.	Cell Biol Int	30	952-956	2006
Ohashi J, Naka I, Toyoda A, Takasu M, Tokunaga K, Ishida T, Sakaki Y, Hohjoh H	Estimation of the species-specific mutation rates of the DRB1 locus in human and chimpanzee.	Tissue Antigens	68	427-431	2006
Kawashima M, Tamiya G, Oka A, Hohjoh H, Juli T, Ebisawa T, Honda Y, Inoko H, Tokunaga K	Genome-wide association analysis of human narcolepsy and a new resistance gene.	Am J Hum Genet	79	252-263	2006
Hachiya NS, Imagawa M, Kaneko K	The possible role of protein X, a putative auxiliary factor in pathological prion replication, in regulating a physiological endoproteolytic cleavage of cellular prion protein	Med Hypotheses		in press	2007
金子清俊	理系の説明責任. BSE問題をめぐって	科学	76	52-55	2006
八谷如美, 金子清俊	プリオン蛋白質異常化の分子機構	化学療法の領域	22	63-68	2006
金子清俊	牛海綿状脳症と変異型クロイツフェルト・ヤコブ病	TMDC MATE	242	12-13	2006
金子清俊	プリオン病 - 牛海綿状脳症 (BSE)と変異型クロイツフェルト・ヤコブ病 -	BRAIN	83	4-5	2006

八谷如美, 金子清俊	正常型プリオンたんぱく質の正体は？ - その推定される機能 -	現代化学	422	26-31	2006
金子清俊	食について考える - 「安全」と「安心」とは -	建設労働の広場	59	52-56	2006
金子清俊, 神田敏子, 水澤英洋, 山内一也	BSEの危険度はどこまでわかったのか -何が問われるべきか-	科学	76	1105-1112	2006
八谷如美, 金子清俊	プリオンたんぱく質は正常人では何をしているのか？	科学	76	1138-1142	2006
桑田一夫	バイオインフォーマティクスによるプリオン病治療薬の開発	化学療法の領域	22	87-93	2006
桑田一夫	連載“話題のウイルス” NO.12 プリオン	Drug Delivery System (DDS)	21	156-157	2006
山口圭一, 松本友治, 児玉耕太, 岸直人, 桑田一夫	プリオン病の発症と伝播機構－特集 アミロイドの謎は解けるか？：プリオン病・アルツハイマー病・透析アミロイドーシスなどの病態を紐解く－	細胞工学	26（2）	151-155	2007
後藤祐児, 桑田一夫, 関島良樹, 田中元雅, 内木宏延, 永井義隆, 松崎勝巳, 樋口京一	アミロイドーシス発症の分子機構解明を目指して：現状と展望，夢－特集 アミロイドの謎は解けるか？：プリオン病・アルツハイマー病・透析アミロイドーシスなどの病態を紐解く－	細胞工学	26（2）	181-185	2007
北條　浩彦	神経・筋疾患治療へのRNAi応用	神経治療学	23	31-36	2006
北條　浩彦	RNAi効果の評価法	バイオテクノロジージャーナル	6	51-57	2006
八谷如美, 金子清俊	正常Prion蛋白の機能と異常化 (感染)のメカニズム	Brain Medical		印刷中	2007
八谷如美, 金子清俊	正常Prion蛋白とその機能	日本臨床		印刷中	2007

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Neuroscience Letters 402 (2006) 222–226
Neuroscience Letters
www.elsevier.com/locate/neulet

Intracerebroventricular delivery of dominant negative prion protein in a mouse model of iatrogenic Creutzfeldt-Jakob disease after dura graft transplantation

Kazuhide Furuya[a,*], Nobutaka Kawahara[a], Yoshio Yamakawa[b], Hitaru Kishida[c], Naomi S. Hachiya[d], Masahiro Nishijima[b], Takaaki Kirino[a], Kiyotoshi Kaneko[d]

[a] *Department of Neurosurgery, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b] *Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan*
[c] *Department of Neurology, Yokohama City University, Yokohama 236-0004, Japan*
[d] *Second Department of Physiology, Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan*

Received 21 February 2006; received in revised form 10 March 2006; accepted 25 March 2006

Abstract

We have developed a novel procedure in which a small collagen sheet (3 mm × 3 mm) absorbing prion-infected brain homogenates was transplanted onto the brain surface of highly prion-susceptible transgenic mice (Tg(MoPrP)4053/FVB), as an animal model of iatrogenic Creutzfeldt-Jakob disease (iCJD) caused by prion-contaminated cadaveric dura graft transplantation. Using the iCJD model, we further investigated the in vivo efficacy of dominant negative recombinant prion protein with lysine substitution at mouse codon 218 (rPrP-Q218K), which is known to inhibit prion replication in vitro (H. Kishida, Y. Sakasegawa, K. Watanabe, Y. Yamakawa, M. Nishijima, Y. Kuroiwa, N.S. Hachiya, K. Kaneko, Non-glycosylphosphatidylinositol (GPI)-anchored recombinant prion protein with dominant-negative mutation inhibits PrPSc replication in vitro, Amyloid, vol. 11, 2004, pp. 14–20.). Following 7-day intracerebroventricular administration of the rPrP-Q218K via an indwelling catheter connected to the implanted osmotic pump, the median incubation period of Tg(MoPrP)4053/FVB was prolonged considerably from 117 days to 131 days ($p = 0.016$, log-rank test) in the rPrP-Q218K-treated group, even after a lengthy latency period of as long as 30 days by starting the rPrP-Q218K injection. Whether wild-type rPrP, other mutant rPrPs, or the combination of rPrP-Q218K with other anti-prion compounds might extend the survival period in that condition must be further investigated.

Keywords: Iatrogenic Creutzfeldt-Jakob disease (iCJD); Animal model of iCJD; Osmotic pump; Highly prion-susceptible transgenic mice (Tg(MoPrP)4053/FVB); Dominant negative recombinant prion protein (rPrP-Q218K)

Prion diseases are a group of neurodegenerative disorders including kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle, which comprise sporadic, genetic, or infectious disorders [22]. The post-translational conformational change of the cellular isoform of prion protein (PrP^C) into the scrapie isoform of prion protein (PrP^{Sc}) is the fundamental process underlying the pathogenesis of prion diseases [23,24].

Among the infectious forms of CJD, iatrogenic CJD (iCJD) is the most representative. In that form, several sources for the iatrogenic prion infection have been reported, including improperly sterilized cortical and depth electrodes, transplanted corneas, cadaveric pituitary-derived human growth hormone/gonadtropin, and dura graft [4]. By the year 2000, 114 cases of iCJD caused by cadaveric dura transplants from CJD patients had been reported worldwide [4]; about two-thirds of the CJD patients who had received cadaveric dura graft transplantation were from Japan [6,12]. That highest incidence in Japan most probably results from extensive use of cadaveric dura graft: it far exceeds the use in any other country [2]. Emerging patients as well as suspected cases, and the increase in the mean and range of the latency period all suggest that this outbreak is ongoing [3]. In spite of its significance and urgent necessity, no

* Corresponding author. Tel.: +81 3 5800 8853; fax: +81 3 5800 8655.
E-mail address: furuya-nsu@umin.ac.jp (K. Furuya).

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2006.03.062

animal model with prion transmission via cortico-dural interface has been verified so far. Consequently, development of an animal model toward exploration of prophylactic and therapeutic approaches is urgent.

Naturally occurring polymorphic PrP variants (Q171R and E219K) are known to render sheep and humans resistant to scrapie and CJD, respectively, and were found to act as dominant negatives [13,33]. Similarly, recombinant mouse PrP with a lysine variant at codon 218 (rPrP-Q218K) corresponding to human E219K, but not wild-type rPrP, exclusively inhibited prion replication as dominant negatives in scrapie-infected mouse neuro2a (ScN2a) cells without reducing cell viability in vitro [13].

With this background, we have developed a mouse model of iCJD after artificial dura graft transplantation. Having established the mouse model for the first time in vivo, we further investigated whether the dominant negative rPrP-Q218K can be protective against experimental iCJD after prion-contaminated dura graft transplantation in vivo.

Transgenic mice with FVB background harboring a high-copy-number of wild-type PrP-A transgenes (Tg(MoPrP-A)B4053/FVB) [32] were kindly supplied by Dr. S.B. Prusiner of UCSF. A mouse-adapted scrapie Obihiro strain [29], a kind gift from Dr. M. Horiuchi of Hokkaido University, was inoculated intracerebrally into the Tg(MoPrP-A)B4053/FVB. Their brains were collected approximately 70 days after inoculation, and 10% (w/v) brain homogenates in phosphate buffer saline (PBS) were prepared and further provided as inocula (30 μl each) using a 27-gauge disposable hypodermic needle inserted into the right parietal lobe. Diagnosis of scrapie in the transgenic mice has been described extensively elsewhere [5,25]. Beginning 50 days after inoculation, the mice and hamsters were examined every 3 days for neurologic dysfunction. Once clinical signs were detected, the animals were inspected daily and sacrificed when death was imminent. Representative fractions of brains were removed for histological analyses to confirm the scrapie diagnosis.

The recombinant PrP (rPrP) was expressed as inclusion bodies in the *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA) in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Inclusion bodies were collected from sonicated lysates by centrifugation at 27,000 × *g* for 10 min, washed three times in Buffer A (2 M urea, 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM β-mercaptoethanol (β-ME), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)), and solubilized in Buffer B (8 M urea, 25 mM Tris–HCl, pH 7.5, 2 mM β-ME, 0.5 mM PMSF). After centrifugation (200,000 × *g*, 30 min), the supernatant was applied to a CM-Sepharose column (Amersham Biosciences Corp., Piscataway, NJ), washed with Buffer B containing 100 mM NaCl and eluted with Buffer B containing 150 mM NaCl. The eluate containing rPrP was applied to a Ni-NTA agarose column (Qiagen Inc., Valencia, CA), washed with Buffer B containing 5 mM imidazol and eluted with Buffer B containing 200 mM imidazol. The eluate was diluted 10-fold in 1 M arginine-HCl, pH 8.0, 1 mM reduced glutathione, 0.8 mM oxidized glutathione and incubated at 4 °C overnight. After incubation at 37 °C for 10 min, the refolded recombinant proteins were concentrated and buffer-changed into PBS using ultrafiltration (Ultrafree-15 centrifugal filter device-Biomax 10 K NMWL membrane; Millipore Co., Bedford, MA). Concentrations of rPrP were calculated by absorbance at 280 nm with a specific absorbance unit of 2.70 [15].

As an animal model of iCJD after prion-contaminated dura graft transplantation, the Tg(MoPrP-A)B4053 mice were anesthetized with ketamine and xylazine. Subsequently, the head of each was fixed to a stereotaxic frame. Following a 4 mm × 4 mm craniectomy on the right skull, a small collagen sheet (3 mm × 3 mm) absorbing either 5 μl of 10% brain homogenates derived from prion-infected mice ($n = 16$) or PBS ($n = 12$) as a control was transplanted on the brain surface of Tg(MoPrP-A)B4053 ($n = 5$ each). All procedures in animals accorded strictly with guidelines for experiments involving experimental animals performed at the National Institute of Infectious Diseases, Japan. These mice were re-anesthetized followed by implantation of osmotic pumps (200 μl, Alzet 2001; Alza Corp., Palo Alto, CA) in the back. The pumps were filled with either 200 μg/ml of rPrP-Q218K at 30 ($n = 7$) or 60 ($n = 9$) days after prion-contaminated dura graft transplantation or PBS ($n = 6$ each). These solutions were then administered intracerebroventricularly via an indwelling catheter connected to the pump at a rate of 1 μ/h for 7 days.

Brain tissue was homogenized in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl (TN-buffer) to 10% (w/v) with Multi-beads Shocker (Yasui Kikai Corp., Tokyo). The homogenate (250 μl, containing 25 mg brain tissue equivalent) was clarified by the addition of equal volume of detergent buffer (TN-buffer containing 4% zwittergent 3-13 and 1% sarkosyl) and 25 μl of 2-butanol. After extensive sonication, the mixture was simultaneously digested with collagenase (250 μg) and DNase I (20 μg) at 37 °C for 30 min. Thereafter, 20 μg of proteinase K (PK) was added to the reaction mixture and incubated for 30 min; PrP^{Sc} was then precipitated by addition of 250 μl of 5:1 mixture of 2-butanol and methanol containing 3 mM phenylmethylsulfonyl fluoride. The precipitates collected by centrifugation at 15,000 rpm for 10 min were dissolved in 100 μl of SDS-PAGE sampling buffer and heated at 100 °C for 5 min.

Five micrograms of total brain homogenates in 10 μl from mice in 30 ($n = 3$), 60 ($n = 3$), 90 ($n = 3$), and 120 ($n = 2$) days after prion-contaminated dura graft transplantation was electrophoresed in 12% polyacrylamide gel (NuPAGE Bis–Tris gel, Invitrogen, Carlsbad, CA) for 60 min at a constant voltage of 200 V. Then, total proteins were transferred onto PVDF membrane at a constant voltage of 25 V for 45 min. Mouse PrP^{Sc} was reacted with a rabbit polyclonal anti-mouse PrP antibody P-8, which was purified from the rabbit serum immunized with the synthetic peptide with mouse PrP residues 94–108 (THNQWN KPSKPKTNML). Next, HRP-linked anti-rabbit IgG (Amersham Biosciences, Uppsala, Sweden) was used as a secondary antibody. Immunoreactivity was detected with an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Uppsala, Sweden) and visualized on autoradiographic film.

Brain tissues ($n = 3$ in 120-day group) were fixed with 10% buffered-formalin for 48–72 h. Mouse PrP^{Sc} was first inacti-

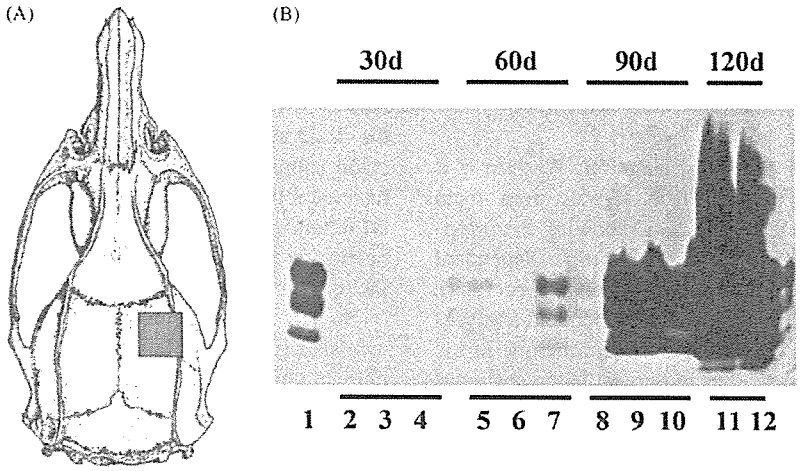

Fig. 1. A mouse model of iatrogenic Creutzfeldt-Jakob disease (iCJD) after prion-contaminated dura graft transplantation. (A) A small collagen sheet (3 mm × 3 mm) containing brain homogenates derived from prion-infected mice was transplanted on the brain surface of highly prion-susceptible transgenic mice (Tg(MoPrP-A)B4053, hatched area). (B) Western blot analyses of Tg(MoPrP-A)B4053 brains at 30, 60, 90 and 120 days (three brains each) after transplantation. Initial detection of PrP^{Sc} in two-thirds of the animals at 60 days (lanes 5, 6 and 7) and strong detection at 90 (lanes 8, 9 and 10) and 120 days (lanes 11 and 12) are observed in a time-dependent manner. Lane 1: Brain homogenates from pooled prion-infected mice as a positive control.

vated by immersing tissues into 98% formic acid for 1 h at room temperature. Samples were re-fixed with the formalin overnight, dehydrated in graded ethanol, and cleared in xylene; then they were embedded in paraffin. Sections cut at 3 μm thickness were mounted on silane-coated slide glasses. After deparaffinization, sections were stained with hematoxylin and eosin. For immunostaining, the deparaffinized sections were autoclaved at 121 °C for 20 min in the presence of 1 mM HCl to destroy PrP^{C} [19]; endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 5 min at room temperature. The sections were treated with 10% normal goat serum in PBS for 5 min. Then they were reacted for 30 min with anti-PrP polyclonal antibody (Ab221–236) [31], which is a rabbit polyclonal anti-bovine PrP purified from the rabbit serum immunized with the synthetic peptide with bovine PrP residues 221–240 (VEQMCITQYQRESQAYYQRG), but cross-reacts with mouse PrP^{C}. The HRP-labeled polymer method (Envision + kit; Dako Cytomation, Carpinteria, CA) was used according to the manufacturer's instructions. Signals were detected using diaminobenzidine (DAB).

The (Tg(MoPrP-A)B4053 harboring a high-copy-number of wild-type PrP-A transgenes were highly susceptible to mouse prions; they exhibited an abbreviated scrapie incubation time of about 45 days after intracerebral inoculation, which is consistent with a previous observation [32]. To establish a mouse model of iCJD after prion-contaminated dura graft transplantation, a small collagen sheet bearing 5 μl of 10% prion-infected mouse brain homogenates was transplanted on the brain surface of Tg(MoPrP-A)B4053 (Fig. 1A). Consequently, all animals died approximately 120 days after the

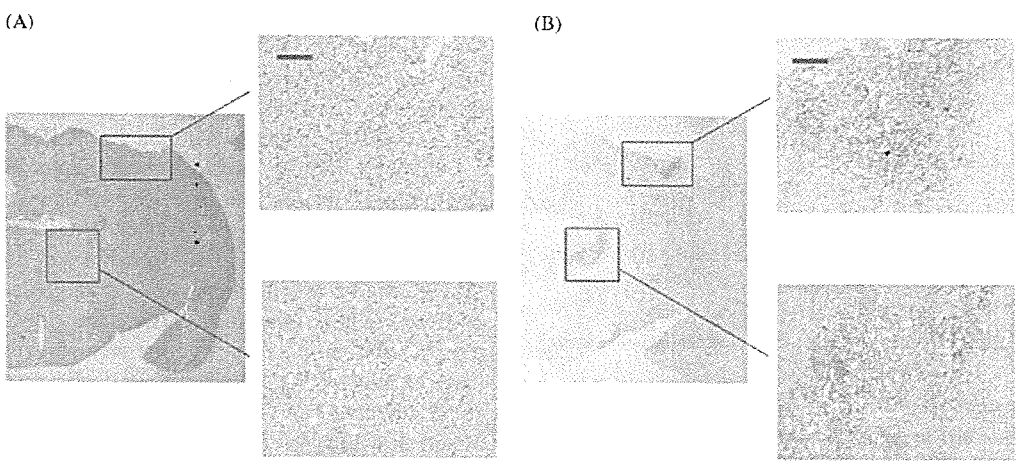

Fig. 2. Accumulation of mouse PrP^{Sc} in brains from the iCJD model at 120 days after prion-contaminated dura graft transplantation revealed by light microscopy and immunohistochemstry. Representative brain sections stained with either hematoxylin or eosin in panel A, or immunostained with anti-PrP antibody (Ab221–236) in panel B. Scale bars = 100 μm.

prion-contaminated dura graft transplantation with two to three times prolonged incubation period compared with those after the intracerebral inoculation, whereas sham-operated or PBS-treated littermates remained healthy for over 300 days after the operation.

Starting at 60 days after the transplantation, Western blot analysis detected discrete mouse PrP^{Sc} signals even from asymptomatic Tg(MoPrP-A)B4053 brains receiving the prion-contaminated dura graft transplantation in a time-dependent manner (Fig. 1B). Concomitant with spongiform degeneration by light microscopy (Fig. 2A), immunohistochemical analyses further disclosed the considerable PrP^{Sc} accumulations in the transplanted cortical areas as well as in the ipsilateral thalamus at 120 days after the transplantation (Fig. 2B), which might support the assumption that the spread of prions occurs axonally after intraocular injection [11].

As a therapeutic and prophylactic approach, we next performed 7-day intracerebroventricular administration of rPrP-Q218K via an indwelling catheter connected to implanted osmotic pump at either 30 or 60 days after prion-contaminated dura graft transplantation. The control group, Tg(MoPrP-A)B4053 treated with PBS alone, died at 117 days after transplantation. To our surprise, the median incubation period of Tg(MoPrP-A)B4053 was markedly prolonged for as long as 131 days even after a long latency period for as long as 30 days by starting the rPrP-Q218K injection (Fig. 3), which was statistically significant compared with that of the control group ($p = 0.016$, log-rank test). After a very long latency period of 60 days by starting the rPrP-Q218K treatment, however, the median incubation period of mice was 113 days, which was not statistically significant (Fig. 3).

For potential iCJD patients who received dura graft transplantations in the past, an effective and efficient prophylactic method has been anticipated. Toward the exploration of such prophylactics, development of an animal model of iCJD after dura graft transplantation is apparently fundamental and crucial.

The intracerebral inoculation of tissue homogenates into suitable recipients is the most effective method for transmission of prion diseases and frequently allows the species barrier to be circumvented. Alternatively, intraperitoneal injection has been used for studies of extra-central nervous system involvement. However, these procedures are unsuitable for investigation of the iCJD after dura graft transplantation because the mode of prion propagation is markedly different. For that reason, we have established for the first time the mouse model of iCJD after artificial dura graft transplantation, in which prion transmission occurs via cortico-dural interface; it has two to three times longer incubation period than that after intracerebral inoculation.

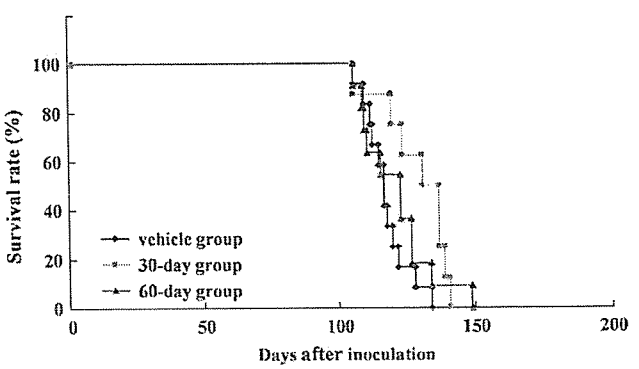

Fig. 3. Kaplan–Meyer survival analysis of highly prion-susceptible Tg(MoPrP-A)B4053 with the dominant negative rPrP-Q218K treatment. The Tg(MoPrP-A)B4053 with the dominant negative rPrP-Q218K treatment started at 30 days after prion-contaminated dura graft transplantation survived significantly longer ($p = 0.016$) than those of the control group.

So far, no effective prophylactic or therapeutic method is available in prion-infected mice during the asymptomatic preclinical phase [7,21] because it is preliminary to consider gene therapeutics, e.g. an ex vivo gene transfer approach [8,14,15]. Furthermore, a recent clinical trial of Aβ vaccination targeting Alzheimer's disease was halted because of the serious neurological complications of autoimmune reactions developing in some patients [10,18].

Human PrP polymorphism at codon 219 has been reported in the Japanese population [16]. Among Japanese, 12% carry the lysine allele, but the lysine allele in place of glutamine has not been found in 85 autopsied sporadic CJD cases, suggesting that the lysine substitution at human codon 219 (E219K), which corresponds to mouse Q218K, might act as a dominant negative form in prion propagation [16,28]. As mentioned, we have reported that the administration of dominant negative rPrP-Q218K sufficiently inhibits PrP^{Sc} formation in ScN2a cells in vitro [15]. For those reasons, further investigation of its therapeutic equivalence must be done in an in vivo setting.

Intrathecal injection is an efficient option for delivery of therapeutic reagents into the brain because the blood–brain barrier (BBB) is often the rate-limiting factor in determining permeation of these reagents in vivo [1,9]. In a preclinical SOD1(G93A) rat model of amyotrophic lateral sclerosis, for example, intracerebroventricular delivery of recombinant vascular endothelial growth factor delays onset of paralysis by 17 days; it also improves motor performance and prolongs survival by 22 days [30]. In humans, clinical applications with continuous intrathecal administration of various therapeutic reagents including bacrofen [17], anti-cancer drugs [20], analgesics [26], and neuroleptics [27] have been used widely and have been found to be effective. These observations further support inferences from our current data in the mouse model of iCJD.

Although limited, intracerebroventricular administration of rPrP-Q218K delayed the onset of prion disease with a longer survival period in the mouse model of iCJD in vivo. A salient advantage is the fact that homogeneous and high-quality rPrP-Q218K can be readily prepared from large-scale fermentation of *E. coli* in large quantities. At the same time, it is also important to introduce wild-type or other mutant rPrPs into the current experimental setting, since the rPrPs itself may interfere with the prion replication in vivo even without the Q218K substitution. Whether wild-type rPrP, other mutant rPrPs, or the combination of rPrP-Q218K with anti-prion compounds might extend the survival period in an in vivo setting remains to be further investigated.

Acknowledgement

This study was supported by grants from the Japanese Ministry of Health, Labour and Welfare and the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

[1] P. Aebischer, M. Schluep, N. Deglon, J.M. Joseph, L. Hirt, B. Heyd, M. Goddard, J.P. Hammang, A.D. Zurn, A.C. Kato, F. Regli, E.E. Baetge, Intrathecal delivery of CNTF using encapsulated genetically modified xenogeneic cells in amyotrophic lateral sclerosis patients, Nat. Med. 2 (1996) 696–699.
[2] T. Blattler, Implications of prion diseases for neurosurgery, Neurosurg. Rev. 25 (2002) 195–203.
[3] C. Boutoleau, B. Guillon, F. Martinez, M. Vercelletto, A. Faure, J.R. Feve, Iatrogenic Creutzfeldt-Jakob disease subsequent to dural graft: persisting risk after, Eur. J. Neurol. 10 (2003) 521–523.
[4] P. Brown, M. Preece, J.P. Brandel, T. Sato, L. McShane, I. Zerr, A. Fletcher, R.G. Will, M. Pocchiari, N.R. Cashman, J.H. d'Aignaux, L. Cervenakova, J. Fradkin, L.B. Schonberger, S.J. Collins, Iatrogenic Creutzfeldt-Jakob disease at the millennium, Neurology 55 (2000) 1075–1081.
[5] G.A. Carlson, D.T. Kingsbury, P.A. Goodman, S. Coleman, S.T. Marshall, S. DeArmond, D. Westaway, S.B. Prusiner, Linkage of prion protein and scrapie incubation time genes, Cell 46 (1986) 503–511.
[6] Y. Nakamura, M. Watanabe, K. Nagoshi, T. Kitamoto, T. Sato, M. Yamada, H. Mizusawa, R. Maddox, J. Sejvar, E. Belay, L.B. Schonberger, Update: Creutzfeldt-Jakob disease associated with cadaveric dura mater grafts–Japan, 1979–2003, MMWR Morb Mortal Wkly Rep 52 (2003) 1179–1181.
[7] S.J. Collins, V. Lewis, M. Brazier, A.F. Hill, A. Fletcher, C.L. Masters, Quinacrine does not prolong survival in a murine Creutzfeldt-Jakob disease model, Ann. Neurol. 52 (2002) 503–506.
[8] S.Y. Corbel, F.M. Rossi, Latest developments and in vivo use of the Tet system: ex vivo and in vivo delivery of tetracycline-regulated genes, Curr. Opin. Biotechnol. 13 (2002) 448–452.
[9] M.E. Cudkowicz, L. Warren, J.W. Francis, K.J. Lloyd, R.M. Friedlander, L.F. Borges, N. Kassem, T.L. Munsat, R.H. Brown Jr., Intrathecal administration of recombinant human superoxide dismutase 1 in amyotrophic lateral sclerosis: a preliminary safety and pharmacokinetic study, Neurology 49 (1997) 213–222.
[10] J.C. Dodart, K.R. Bales, S.M. Paul, Immunotherapy for Alzheimer's disease: will vaccination work? Trends Mol. Med. 9 (2003) 85–87.
[11] H. Fraser, Neuronal spread of scrapie agent and targeting of lesions within the retino-tectal pathway, Nature 295 (1982) 149–150.
[12] K. Hoshi, H. Yoshino, J. Urata, Y. Nakamura, H. Yanagawa, T. Sato, Creutzfeldt-Jakob disease associated with cadaveric dura mater grafts in Japan, Neurology 55 (2000) 718–721.
[13] K. Kaneko, L. Zulianello, M. Scott, C.M. Cooper, A.C. Wallace, T.L. James, F.E. Cohen, S.B. Prusiner, Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 10069–10074.
[14] M.H. Kapturczak, T. Flotte, M.A. Atkinson, Adeno-associated virus (AAV) as a vehicle for therapeutic gene delivery: improvements in vector design and viral production enhance potential to prolong graft survival in pancreatic islet cell transplantation for the reversal of type 1 diabetes, Curr. Mol. Med. 1 (2001) 245–258.
[15] H. Kishida, Y. Sakasegawa, K. Watanabe, Y. Yamakawa, M. Nishijima, Y. Kuroiwa, N.S. Hachiya, K. Kaneko, Non-glycosylphosphatidylinositol (GPI)-anchored recombinant prion protein with dominant-negative mutation inhibits PrPSc replication in vitro, Amyloid 11 (2004) 14–20.
[16] T. Kitamoto, J. Tateishi, Human prion diseases with variant prion protein, Philos. Trans. R. Soc. Lond. B 343 (1994) 391–398.
[17] A.I. Korenkov, W.R. Niendorf, N. Darwish, E. Glaeser, M.R. Gaab, Continuous intrathecal infusion of baclofen in patients with spasticity caused by spinal cord injuries, Neurosurg. Rev. 25 (2002) 228–230.
[18] P.L. McGeer, E. McGeer, Is there a future for vaccination as a treatment for Alzheimer's disease? Neurobiol. Aging 24 (2003) 391–395.
[19] T. Muramoto, T. Kitamoto, J. Tateishi, I. Goto, The sequential development of abnormal prion protein accumulation in mice with Creutzfeldt-Jakob disease, Am. J. Pathol. 140 (1992) 1411–1420.
[20] H. Nakagawa, E. Miyahara, T. Suzuki, K. Wada, M. Tamura, Y. Fukushima, Continuous intrathecal administration of 5-fluoro-2'-deoxyuridine for the treatment of neoplastic meningitis, Neurosurgery 57 (2005) 266–280 (discussion 266–280).
[21] S.A. Priola, A. Raines, W.S. Caughey, Porphyrin and phthalocyanine antiscrapie compounds, Science 287 (2000) 1503–1506.
[22] S.B. Prusiner, D.C. Bolton, D.F. Groth, K.A. Bowman, S.P. Cochran, M.P. McKinley, Further purification and characterization of scrapie prions, Biochemistry 21 (1982) 6942–6950.
[23] S.B. Prusiner, P. Peters, K. Kaneko, A. Taraboulos, V. Lingappa, F.E. Cohen, S.J. DeArmond, Cell Biology of Prions, Cold Spring Harbor, 1999, pp. 349–391.
[24] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 13363–13383.
[25] S.B. Prusiner, M. Scott, D. Foster, K.-M. Pan, D. Groth, C. Mirenda, M. Torchia, S.-L. Yang, D. Serban, G.A. Carlson, P.C. Hoppe, D. Westaway, S.J. DeArmond, Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication, Cell 63 (1990) 673–686.
[26] N.G. Rainov, V. Heidecke, W. Burkert, Long-term intrathecal infusion of drug combinations for chronic back and leg pain, J. Pain Symptom Manage. 22 (2001) 862–871.
[27] S. Seki, K. Sasaki, M.O. Fraser, Y. Igawa, O. Nishizawa, M.B. Chancellor, W.C. de Groat, N. Yoshimura, Immunoneutralization of nerve growth factor in lumbosacral spinal cord reduces bladder hyperreflexia in spinal cord injured rats, J. Urol. 168 (2002) 2269–2274.
[28] S. Shibuya, J. Higuchi, R.W. Shin, J. Tateishi, T. Kitamoto, Protective prion protein polymorphisms against sporadic Creutzfeldt-Jakob disease (letter), Lancet 351 (1998) 419.
[29] M. Shinagawa, K. Takahashi, S. Sasaki, S. Doi, H. Goto, G. Sato, Characterization of scrapie agent isolated from sheep in Japan, Microbiol. Immunol. 29 (1985) 543–551.
[30] E. Storkebaum, D. Lambrechts, M. Dewerchin, M.P. Moreno-Murciano, S. Appelmans, H. Oh, P. Van Damme, B. Rutten, W.Y. Man, M. De Mol, S. Wyns, D. Manka, K. Vermeulen, L. Van Den Bosch, N. Mertens, C. Schmitz, W. Robberecht, E.M. Conway, D. Collen, L. Moons, P. Carmeliet, Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS, Nat. Neurosci. 8 (2005) 85–92.
[31] H. Takahashi, R.H. Takahashi, H. Hasegawa, M. Horiuchi, M. Shinagawa, T. Yokoyama, K. Kimura, M. Haritani, T. Kurata, K. Nagashima, Characterization of antibodies raised against bovine-PrP-peptides, J. Neurovirol. 5 (1999) 300–307.
[32] G.C. Telling, T. Haga, M. Torchia, P. Tremblay, S.J. DeArmond, S.B. Prusiner, Interactions between wild-type and mutant prion proteins modulate neurodegeneration in transgenic mice, Genes Dev. 10 (1996) 1736–1750.
[33] L. Zulianello, K. Kaneko, M. Scott, S. Erpel, D. Han, F.E. Cohen, S.B. Prusiner, Dominant-negative inhibition of prion formation diminished by deletion mutagenesis of the prion protein (In Process Citation), J. Virol. 74 (2000) 4351–4360.

Medical Hypotheses (2006) 67, 169–171

ELSEVIER

medical hypotheses

http://intl.elsevierhealth.com/journals/mehy

The alternative role of 14-3-3 zeta as a sweeper of misfolded proteins in disease conditions

Kiyotoshi Kaneko *, Naomi S. Hachiya

Second Department of Physiology, Tokyo Medical University, 6-1-1 Shinjuku, Tokyo 160-8402, Japan

Received 5 January 2006; accepted 10 January 2006

Summary Here, we propose a novel hypothesis that 14-3-3 zeta might act as a sweeper of misfolded proteins by facilitating the formation of aggregates, which are referred to as inclusion bodies. Studies on the localization of the 14-3-3 proteins in different types of inclusion bodies in the brain including neurofibrillary tangle in Alzheimer's disease, pick bodies in Pick's disease, Lewy body-like hyaline inclusions in sporadic amyotrophic lateral sclerosis, prion/florid plaques in sporadic/variant Creutzfeldt–Jakob disease, nuclear inclusions in spinocerebellar ataxia-1, and possibly Lewy bodies in Parkinson's disease suggest a close association of these diseases with 14-3-3 zeta. The highly conserved hydrophobic surface of the amphipathic groove in 14-3-3 zeta represents a general mechanism with diverse cellular proteins, and it may also allow for the molecular recognition of misfolded proteins by hydrophobic interaction in disease conditions. When the abnormal processing of misfolded proteins overwhelms the quality control systems of the cell, it is likely that 14-3-3 zeta is recruited to form deposits of protein aggregates with nonnative, misfolded proteins in order to protect the cell against toxicity. Hence, 14-3-3 zeta may be considered as an auxiliary therapeutic tool in the protein aggregation disorders.

The seven known members of the 14-3-3 family are highly conserved eukaryotic proteins that modulate the action of other proteins that are involved in cell cycle, transcriptional control, signal transduction, intracellular trafficking, and regulation of ion channels. This occurs through to their multiple interactions with various kinases, receptors, enzymes, and structural and cytoskeletal proteins in a phosphorylation-dependent manner [1].

Despite such significant understandings in the physiological roles of 14-3-3 proteins, the possible role of these proteins in disease conditions remains unknown. Here, we propose a novel hypothesis that 14-3-3 zeta might act as a sweeper of misfolded proteins by facilitating the formation of aggregates, which are referred to as inclusion bodies.

A wide variety of neurodegenerative diseases, such as Alzheimer's disease [2], Parkinson's disease [3], Pick's disease [4], amyotrophic lateral sclerosis (ALS), Huntington's disease, and prion diseases including sporadic Creutzfeldt–Jakob disease (CJD) [5] are characterized by the accumulation of intracellular or extracellular protein aggregates (inclusion bodies) [6].

Studies on the localization of the 14-3-3 proteins in different types of inclusion bodies in the brain in

* Corresponding author. Tel.: +81 3 3351 6141x322; fax: +81 3 3351 6544.
E-mail address: k-kaneko@tokyo-med.ac.jp (K. Kaneko).

0306-9877/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mehy.2006.01.019

Table 1 Protein aggregates and 14-3-3 isoforms

Protein aggregates	Substrates	14-3-3 (isoforms)
NFTs[a] in AD[b]	Tau	+ (Beta, zeta)
PBs[c] in PD[d]	Tau	+ (Beta, gamma, epsilon, eta, zeta)
LBHIs[e] in ALS[f]	Unknown	+ (Beta, gamma, theta, eta, zeta)
NIs[g] in SCA-1[h]	Ataxin-1	+ (Eta, zeta)
Prion plaque in sCJD[i]	PrP^{Sc} [j]	+ (Zeta)
Florid plaque in vCJD[k]	PrP^{Sc} [j]	+ (Zeta)
CSF[l] control in sCJD[i]	CSF[l]	+ (Beta, gamma, epsilon, eta)

[a] NFT, neurofibrillary tangles.
[b] AD, Alzheimer's disease.
[c] PBs, pick bodies.
[d] PD, Pick's disease.
[e] LBHIs, Lewy body-like hyaline inclusions.
[f] ALS, amyotrophic lateral sclerosis.
[g] NIs, nuclear inclusions.
[h] SCA-1, spinocerebellar ataxia-1.
[i] sCJD, sporadic Creutzfeldt–Jakob disease.
[j] PrP^{Sc}, scrapie (infectious) isoform of prion protein.
[k] vCJD, variant Creutzfeldt–Jakob disease.
[l] CSF, cerebrospinal fluid.

Alzheimer's disease [7–9], Pick's disease [10], sporadic ALS [11], sporadic/variant CJD [12], and spinocerebellar ataxia-1 (SCA-1) suggest a close association of these diseases with 14-3-3 zeta (Table 1) [7–14]. Although a distinct pattern of the 14-3-3 isoform is yet to be characterized, Lewy bodies observed in Parkinson's disease [15] and the numerous spheroids observed in sporadic ALS [11] showed intense immunolabeling for 14-3-3.

The crystal structure of 14-3-3 zeta reveals a conserved amphipathic groove that represents a general mechanism for the interaction of 14-3-3 proteins with diverse cellular proteins [16]. In fact, serotonin *N*-acetyltransferase is bound in the central channel of the 14-3-3 zeta dimer and is held in place by extensive interactions with both the amphipathic phosphopeptide-binding groove of 14-3-3 zeta and with other parts of the central channel [17].

The highly conserved hydrophobic surface of the amphipathic groove in 14-3-3 zeta, which implies its general importance in ligand binding, may allow for the molecular recognition of misfolded proteins by hydrophobic interaction in a similar fashion that most conventional molecular chaperones recognize and bind to the hydrophobic surfaces of nonnative, misfolded proteins [18]. When the abnormal processing of misfolded proteins overwhelms the quality control systems of the cell, it is likely that 14-3-3 zeta is recruited to form deposits of protein aggregates in order to protect the cell against toxicity [19]. In this regard, direct or indirect application of 14-3-3 zeta through biochemical or gene therapeutic approaches may be considered as an auxiliary therapeutic tool in the protein aggregation disorders.

The capability of forming heterodimers with other 14-3-3 isoforms is a unique characteristic of 14-3-3 zeta, which distinguishes it from other molecules involved in quality control. Since 14-3-3 proteins also appear to control the subcellular localization of ligand proteins [1], the counterpart of 14-3-3 zeta in a heterodimer complex may allow for trafficking in the nucleus, cytosol, and/or the extracellular space [20]. This also appears to be an attractive hypothesis that is yet to be elucidated.

References

[1] Berg D, Holzmann C, Riess O. 14-3-3 proteins in the nervous system. Nat Rev Neurosci 2003;4:752–62.
[2] Lustbader JW, Cirilli M, Lin C, et al. ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. Science 2004;304:448–52.
[3] Greenamyre JT, Hastings TG. Biomedicine. Parkinson's – divergent causes, convergent mechanisms. Science 2004;304:1120–2.
[4] Hachiya NS, Ohkubo T, Kozuka Y, et al. More than a 100-fold increase in immunoblot signals of laser-microdissected inclusion bodies with an excessive aggregation property by oligomeric actin interacting protein 2/d-lactate dehydrogenase protein 2. Anal Biochem 2005;347:106–11.
[5] Mayer MP, Schroder H, Rudiger S, et al. Multistep mechanism of substrate binding determines chaperone activity of Hsp70. Nat Struct Biol 2000;7:586–93.
[6] Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nat Med 2004;10(Suppl.):S10–7.
[7] Hashiguchi M, Sobue K, Paudel HK. 14-3-3zeta is an effector of tau protein phosphorylation. J Biol Chem 2000; 275:25247–54.
[8] Hernandez F, Cuadros R, Avila J. Zeta 14-3-3 protein favours the formation of human tau fibrillar polymers. Neurosci Lett 2004;357:143–6.
[9] Umahara T, Uchihara T, Tsuchiya K, et al. 14-3-3 proteins and zeta isoform containing neurofibrillary tangles in patients with Alzheimer's disease. Acta Neuropathol (Berl) 2004;108:279–86.
[10] Umahara T, Uchihara T, Tsuchiya K, et al. Immunolocalization of 14-3-3 isoforms in brains with Pick body disease. Neurosci Lett 2004;371:215–9.
[11] Kawamoto Y, Akiguchi I, Nakamura S, Budka H. 14-3-3 proteins in Lewy body-like hyaline inclusions in patients with sporadic amyotrophic lateral sclerosis. Acta Neuropathol (Berl) 2004;108:531–7.
[12] Richard M, Biacabe AG, Streichenberger N, et al. Immunohistochemical localization of 14.3.3 zeta protein in amyloid plaques in human spongiform encephalopathies. Acta Neuropathol (Berl) 2003;105:296–302.
[13] Chen HK, Fernandez-Funez P, Acevedo SF, et al. Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell 2003;113:457–68.

[14] Wiltfang J, Otto M, Baxter HC, et al. Isoform pattern of 14-3-3 proteins in the cerebrospinal fluid of patients with Creutzfeldt–Jakob disease. J Neurochem 1999;73:2485–90.
[15] Kawamoto Y, Akiguchi I, Nakamura S, et al. 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains. J Neuropathol Exp Neurol 2002;61:245–53.
[16] Liu D, Bienkowska J, Petosa C, et al. Crystal structure of the zeta isoform of the 14-3-3 protein. Nature 1995;376:191–4.
[17] Obsil T, Ghirlando R, Klein DC, Ganguly S, Dyda F. Crystal structure of the 14-3-3 zeta:serotonin *N*-acetyltransferase complex. A role for scaffolding in enzyme regulation. Cell 2001;105:257–67.
[18] Houry WA. Mechanism of substrate recognition by the chaperonin GroEL. Biochem Cell Biol 2001;79:569–77.
[19] Tanaka M, Kim YM, Lee G, et al. Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. J Biol Chem 2004;279:4625–31.
[20] Forman MS, Trojanowski JQ, Lee VM. Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. Nat Med 2004;10:1055–63.

Biotechnol Lett
DOI 10.1007/s10529-006-9106-7

ORIGINAL PAPER

A screening method for DNA aptamers that bind to a specific, unidentified protein in tissue samples

Takahisa Noma · Kazunori Ikebukuro · Koji Sode · Takuya Ohkubo · Yuji Sakasegawa · Naomi Hachiya · Kiyotoshi Kaneko

Received: 24 March 2006 / Accepted: 8 May 2006

Abstract Aptamers are oligonucleotide ligands with a high affinity to, and specificity for, various target molecules and they are expected to be powerful tools for proteomic analysis. To select aptamers that bind to a specific unidentified protein in tissues for protein analysis, a screening method was developed using chicken skeletal muscle as a model. Target proteins in the target mixture were separated by electrophoresis and transferred to a membrane, and a DNA library was added onto it. The aptamers that bound to the target protein were visualized by chemiluminescence and collected by cutting out the visualized band. The specific aptamers to the target protein were selected by only one round of selection using this screening, suggesting this screening method might be useful for selecting aptamers for proteome analysis.

T. Noma · K. Ikebukuro (✉) · K. Sode
Department of Life Science and Biotechnology, Tokyo University of Agriculture of and Technology, 2-24-16, Naka-cho, Koganei, Tokyo 184-8588, Japan
e-mail: ikebu@cc.tuat.ac.jp

T. Ohkubo · Y. Sakasegawa · N. Hachiya · K. Kaneko
Second Department Physiology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku, Tokyo 160-8402, Japan

Keywords Aptamer · Electrophoresis blotting · Multiple target proteins · Systematic evolution of ligands by exponential enrichment

Introduction

Aptamers are oligonucleotide ligands that bind to various target molecules including proteins with a high affinity and specificity (Gold et al. 1995, Osborne and Ellington 1997). Since they are nucleic acids, they can be easily synthesized chemically and modified for protein detection. Therefore, aptamers are expected to be useful for protein analysis and potential ligands comparable to antibodies.

A purified single target is usually used in the selection of aptamers but screening using complex targets, such as crude solution, cells, or tissue, has advantages since aptamers can be selected without the purification of target proteins (Bianchini et al. 2001, Morris et al. 1998). Also, if aptamers that bind to unidentified target proteins are obtained, such aptamers would become very powerful tools for the identification of those proteins (Blank et al. 2001).

There were several attempts for screening aptamers to proteins in complex targets. However, such screening is still difficult since it is not certain whether the aptamers to the target proteins are obtained during selection. To test this, the selection should be completed, and each

oligonucleotide in a selected library should be sequenced. Then, those oligonucleotides should be synthesized and assayed using various methods, such as a combination of affinity chromatography or photo-crosslinking assay with SDS-PAGE, or Western blotting using aptamers (Daniels et al. 2003, Wang et al. 2000). However, these methods are laborious and time-consuming and, if selected, aptamers do not bind to a supposedly important target protein, the selection must be performed again.

To solve this problem, a screening method to select aptamers to a specific target protein in complex targets is reported in this study (Fig. 1). In this method, the binding of oligonucleotides in the library to each individual protein in complex targets is visualized using a detection method we call aptamer blotting (Fig. 1A). Then, oligonucleotides that bind to a target protein are isolated by cutting out the visualized band (Fig. 1B).

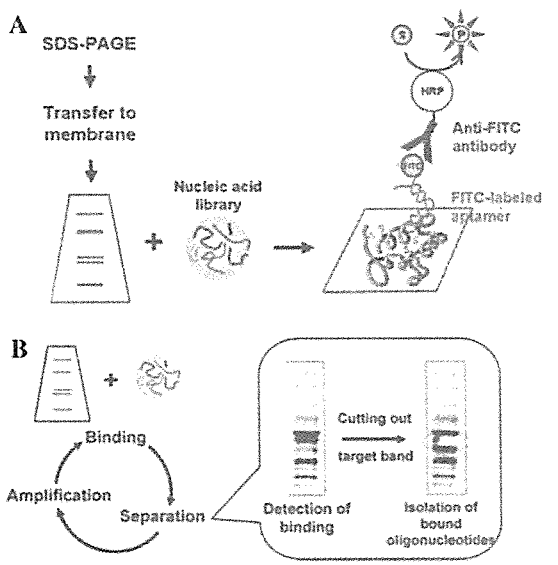

Fig. 1 The scheme of aptamer blotting and the screening using aptamer blotting. (**A**) The scheme of aptamer blotting. The proteins that were separated by electrophoresis and transferred to a membrane were incubated with the FITC-labeled libraries or aptamers. To detect bindings of the oligonucleotides in the library to each individual protein in the target mixture, anti-FITC antibody conjugated with HRP was added and the generated chemiluminescence was detected. (**B**) The scheme of the screening using aptamer blotting. The bound oligonucleotides in a library to the specific target protein, Msc35, were isolated by cutting out the corresponding band after aptamer blotting

Materials and methods

Oligonucleotides

Oligonucleotides and those labeled with FITC were synthesized by a standard solid-phase chemical synthesis and purchased from Invitrogen (Tokyo, Japan). A single-stranded DNA (ssDNA) pool containing 30 randomized bases (5′- FITC-GTA CCA GCT TAT TCA ATT-N30-AGA TAG TAT GTT CAT CAG-3′) was used for screening. This pool was amplified using a forward primer (5′-GTA CCA GCT TAT TCA ATT-3′) and a reverse primer (5′-CTG ATG AAC ATA CTA TCT-3′). The sequence of anti-Tenascin-C aptamer, which was used for a control experiment, is 5′-GGC TGT TGT GAG CCT CCT CCC AGA GGG AAG ACT TTA GGT TCG GTT CAC GTC CCG CTT ATT CTT ACT-3′ (Daniels et al. 2003). The original 69-mer anti-Tenascin-C aptamer was truncated by three cytosines at 3′ end of this aptamer were truncated to render it the same length as the other aptamers used in this study. The secondary structure of the truncated aptamer did not change according to the structure prediction algorithm (Zuker 2003).

Screening using aptamer blotting

A tissue block of chicken skeletal muscle was purchased from Funakoshi (Tokyo, Japan). After the addition of four volumes of PBS (20 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl) to the tissue block (about 2 g), the muscle homogenate was prepared by mincing it with a pair of scissors and a homogenizer followed by the centrifugation.

The scheme of selection using aptamer blotting is shown in Fig. 1. This scheme of selection referred to experimental conditions of conventional standard Western blotting and the aforementioned similar experiments using aptamers (Bianchini et al. 2001; Murphy et al. 2003). The muscle homogenate containing 19 μg protein was dissolved in sample buffer for SDS-PAGE (63 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue, 1% (v/v) 2-mercaptoethanol) and separated on 10% (v/v) homogeneous gel. It was then

transferred to a nitrocellulose membrane. The membrane was blocked with 4% (w/v) skim milk in PBSTE (PBS containing 0.05% (v/v) Tween 20 and 1 mM EDTA) and left overnight at 4°C. The FITC-labeled single-stranded DNA library was dissolved in PBS, heated at 94°C for 3 min, and then gradually cooled to room temperature at a rate of 3°C per min. This library solution, at a final concentration of 10 nM, was incubated with the separated proteins on the membrane for 1 h in PBSTE. After washing with PBSTE four times, the membrane was incubated with 1,000-fold diluted anti-FITC antibody HRP conjugated (Dako, Kyoto, Japan) in PBSTE for 1 h. After washing the membrane with PBSTE as mentioned above, the blots were visualized with ECL Plus Western blotting system (Amersham Biosciences, Tokyo, Japan) (Fig. 1A). The part of the membrane, where the band corresponding to the target protein was supposed to be there, was cut out without the addition of ECL Plus by referring to the detected bands on another membrane treated with ECL Plus (Fig. 1B), since chemiluminescent reagents in ECL-Plus seemed to inhibit PCR reaction. After cutting out the membrane, the membrane was briefly washed with PBSTE and extracted with phenol, and then the supernatant was precipitated with ethanol.

The collected oligonucleotides were amplified by PCR. The PCR solution contained 0.2 mM dNTPs, 0.4 μM forward and reverse primers, and 25 U/ml Ampli*Taq* Gold (Applied Biosystems, Tokyo, Japan). The thermal cycle was as follows: initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and final extension at 72°C for 3 min. Then, the collected oligonucleotides were subcloned by pGEM-T Vector Systems (Promega) and sequenced.

Results

Screening using aptamer blotting and muscle tissue as a model

To develop a method to select aptamers to a specific target protein in complex targets, the procedure of aptamer blotting was developed (Fig. 1). In this study, chicken skeletal muscle was used as a model, since muscle cells contain relatively small numbers of proteins, and the amount of each protein component contained in this tissue was well characterized (Ohtsuki et al. 1986). First, proteins in complex targets, the muscle homogenate, were separated by SDS-PAGE and transferred to a membrane (Fig. 2, lane 2). A major band at near 45 kDa would be actin, which accounts for 20% of the total weight of muscle protein (Ohtsuki et al. 1986). Then, the ssDNA library was incubated with muscle proteins on a membrane, and the bindings of oligonucleotides in the library were visualized (Fig. 2, lane 3). As this figure shows, multiple bands were observed without significant binding to any nitrocellulose membrane. Also, these bands were not observed on the membrane when the ssDNA library was incubated with the muscle homogenate digested with a protease (data not shown). This suggested that the aptamers that bound to the proteins in the muscle homogenate were contained in the initial library to some extent, and the binding of such aptamers to each individual protein in the complex targets was actually monitored by aptamer blotting.

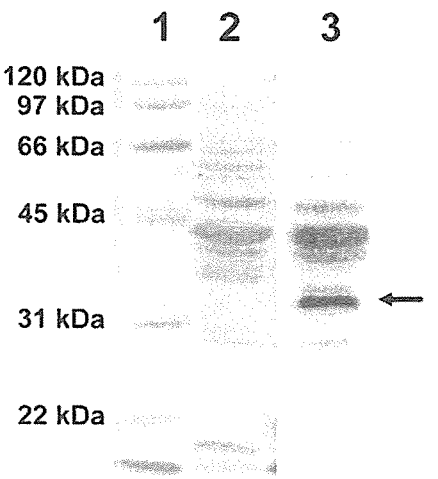

Fig. 2 The binding patterns of the initial library and the selected aptamer. In lane 1 and 2, protein marker and muscle homogenate were stained with Ponceau S, followed by SDS-PAGE and transferred to a membrane. Muscle proteins were probed with the initial library before the screening (lane 3). An arrow indicates the band came from the target protein, Msc35

Many oligonucleotides in the initial library bound to actin, which is a major protein in muscle. Interestingly, a number of oligonucleotides also bound to a protein at near 35 kDa (Fig.2, lane 3), although no clear bands were observed at near 35 kDa on a membrane where muscle proteins were stained (Fig. 2, lane 2). There was no report on such a protein which seems to bind DNAs with a molecular weight of 35 kDa to our knowledge so that we can say this is an unidentified protein. Therefore, we chose this protein as a specific unidentified target protein in complex targets and called it Msc35, after considering its size. We expected to obtain Msc35-specific aptamers by recovering the oligonucleotides from the corresponding band after aptamer blotting. Hence, the recovered oligonucleotides were subcloned and sequenced after just one round of selection.

Characterization of selected aptamers

Forty clones of the recovered oligonucleotides from the target band were sequenced, and one major group was identified based on multiple representation of a particular motif (Fig. 3A). To characterize and identify the target specific aptamers, aptamer blotting was performed again. As shown in Fig. 3B, the selected aptamer, AB4, bound to the target protein. In this experiment, the concentration of the aptamers was 1 nM; therefore, the affinity of the selected aptamer might be considered to be strong. In contrast, control oligonucleotides, constant sequences of the library at both termini, poly A with all its bases at the random 30-mer region of the library substituted by adenine and anti-Tenascin C aptamer (Daniels et al. 2003) did not bind to Msc35. Thus, the aptamers that tightly and specifically bound to the target protein in the complex targets were successfully selected by the screening method using aptamer blotting.

Discussion

Although a similar selection strategy was used for the selection of phage antibodies (Furuta et al. 2002, Liu et al. 2002), and Bianchini *et al.* applied similar manipulation to aptamer blotting to select aptamers (Bianchini et al. 2001), this is the first report that aptamer blotting was applied to the selection of aptamers to the specific unidentified target proteins in complex targets. Bianchini's method aimed to select aptamers from the defined target proteins, but our method aims to select aptamers from arbitrary unidentified proteins; therefore, the aims of screening and application of selected aptamers are very different. In the case of Bianchini's method, the target protein must be determined in advance. Moreover, the cell lysate in which the target protein was highly expressed was necessary for the selection. However, in proteomic analysis, the selection of ligands to unidentified proteins whose functions are not well characterized is important, since such ligands can be useful for the analysis of such target proteins. Therefore, our selection method

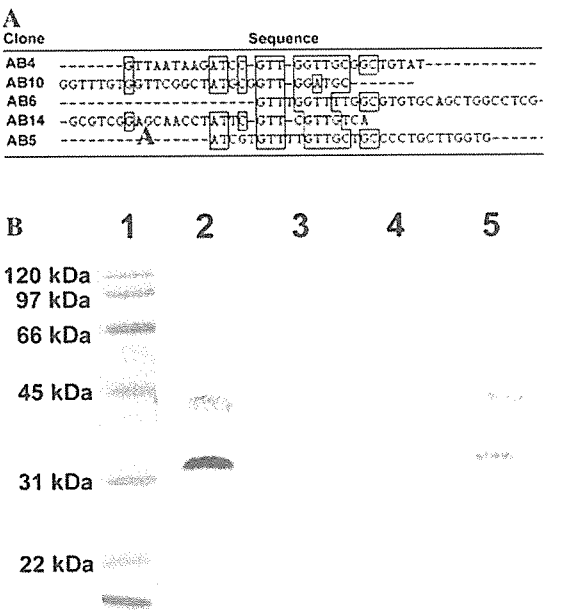

Fig. 3 Characterization of the selected aptamer. (**A**) The sequences of the family of oligonucleotides recovered from the target band. The conserved sequences among oligonucleotides in the family are boxed. (**B**) The binding patterns of selected aptamer to the target protein. Protein marker on the membrane stained with Ponceau S (lane 1). Muscle proteins were probed with the selected aptamer, AB4 (lane 2), constant sequences of the library at both termini (lane 3), poly A with all its bases at the random 30-mer region of the library were substituted by adenine (lane 4), and anti-Tenascin C aptamer (lane 5)

using aptamer blotting would be useful for proteomic analysis.

In order to select aptamers to a specific target protein in complex targets, a novel screening method using aptamer blotting and muscle tissue as a model was developed and evaluated. The aptamers that specifically bound to the specific protein in the muscle were successfully identified by this method without a laborious purification process, suggesting that the selection method using aptamer blotting is efficient in the selection of aptamers as tools for proteome analysis.

Acknowledgement This work is partly supported by the Grant-in-Aid for the 21st Century COE "Future Nano-Materials" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

References

Bianchini M, Radrizzani M, Brocardo MG, Reyes GB, Gonzalez Solveyra C, Santa-Coloma TA (2001) Specific oligobodies against ERK-2 that recognize both the native and the denatured state of the protein. J Immunol Meth 252:191–197

Blank M, Weinschenk T, Priemer M, Schluesener H (2001) Systematic evolution of a DNA aptamer binding to rat brain tumor microvessels. Selective targeting of endothelial regulatory protein pigpen. J Biol Chem 276:16464–16468

Daniels DA, Chen H, Hicke BJ, Swiderek KM, Gold L (2003) A tenascin-C aptamer identified by tumor cell SELEX: systematic evolution of ligands by exponential enrichment. Proc Natl Acad Sci USA 100:15416–15421

Furuta M, Ito T, Eguchi C, Tanaka T, Wakabayashi-Takai E, Kaneko K (2002) Two-dimensional electrophoresis/phage panning (2D-PP): a novel technology for direct antibody selection on 2-D blots. J Biochem 132:245–251

Gold L, Polisky B, Uhlenbeck O, Yarus M (1995) Diversity of oligonucleotide functions. Annu Rev Biochem 64:763–797

Liu B, Huang L, Sihlbom C, Burlingame A, Marks JD (2002) Towards proteome-wide production of monoclonal antibody by phage display. J Mol Biol 315:1063–1073

Morris KN, Jensen KB, Julin CM, Weil M, Gold L. (1998) High affinity ligands from in vitro selection: complex targets. Proc Natl Acad Sci USA 95:2902–7

Murphy MB, Fuller ST, Richardson PM, Doyle SA (2003) An improved method for the in vitro evolution of aptamers and applications in protein detection and purification. Nucleic Acids Res 31:e110

Ohtsuki I, Maruyama K, Ebashi S (1986) Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. Adv Protein Chem 38:1–67

Osborne SE, Ellington AD (1997) Nucleic acid selection and the challenge of combinatorial chemistry. Chem Rev 97:349–370

Wang J, Jiang H, Liu F (2000) In vitro selection of novel RNA ligands that bind human cytomegalovirus and block viral infection. RNA 6:571–583

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31:3406–3415