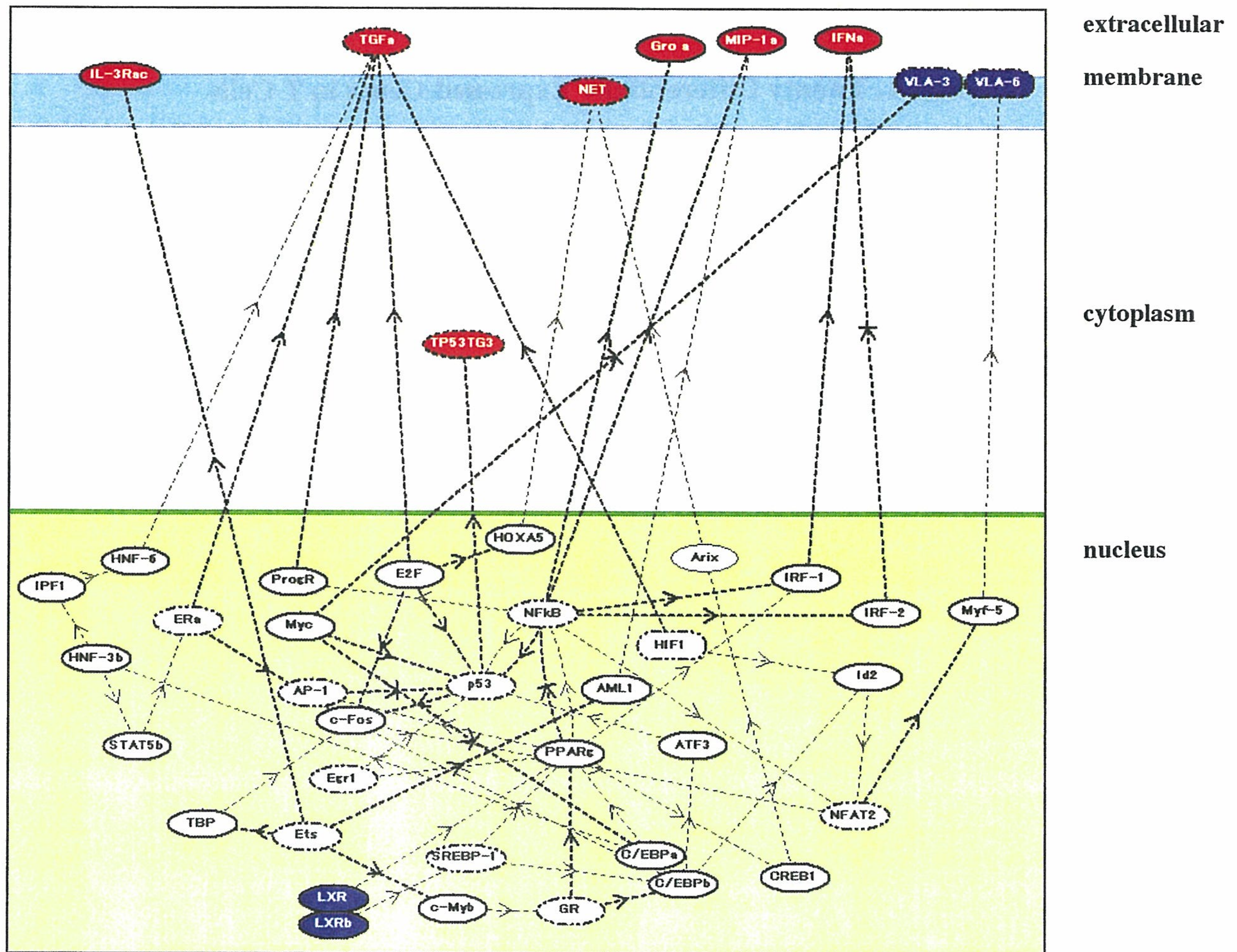

Fig. 1. The common upstream search of 20 differentially expressed genes in T cells between the discordant MS twin. The microarray data of 20 DEG in T cells between the discordant MS twin imported into KeyMolnet extracted 44 genes directly linked to 20 DEG. The "common upstream" search of 44 genes generated a molecular network composed of 43 nodes arranged according to the subcellular distribution. Red nodes represent upregulated genes, whereas purple nodes represent downregulated genes in the MS patient. White nodes exhibit the genes extracted by KeyMolnet to establish molecular connections. The direction of molecular relation is indicated by dash line with arrow (transcriptional activation) or dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet **(Satoh et al. Submitted for publication)**.

Table 2. 58 MS Diagnostic Genes (MDG) of T Cells

No.	Gene Symbol	GenBank Accession Number (RefSeq)	MS-A vs Nc Ratio	No.	Gene Symbol	GenBank Accession Number (RefSeq)	MS-A vs Nc Ratio
1	HSPA1A	NM_005345	0.281	30	PSMC6	NM_002806	0.553
2	ABCB6	NM_005689	0.531	31	PEMT	NM_007169	1.822
3	DOK1	NM_001381	0.54	32	CYP17A1	NM_000102	1.75
4	ZEB1	NM_030751	3.214	33	CDC42	NM_001791	1.742
5	CHST2	NM_004267	0.537	34	TOP1	NM_003286	0.608
6	PTPN6	NM_002831	0.491	35	RASSF7	NM_003475	0.638
7	GADD45A	NM_001924	2.678	36	HBEGF	NM_001945	2.085
8	NR4A2	NM_006186	3.115	37	TNFAIP3	NM_006290	2.034
9	NFKB2	NM_002502	2.59	38	GABPB2	NM_002041	0.609
10	TPST2	NM_003595	0.526	39	PARP1	NM_001618	0.619
11	CCR5	NM_000579	0.355	40	CDC25A	NM_001789	1.863
12	NFKBIA	NM_020529	2.023	41	BAG1	NM_004323	0.694
13	SGK	NM_005627	2.44	42	POLR2H	NM_006232	0.656
14	IRF2	NM_002199	0.546	43	MCM3	NM_002388	0.57
15	ATF3	NM_001674	2.853	44	ATP6V1C1	NM_001695	0.617
16	SLC35A1	NM_006416	0.549	45	MST1R	NM_002447	0.688
17	CDK4	NM_000075	0.591	46	ALDH9A1	NM_000696	0.617
18	BRCA1	NM_007294	2.15	47	TNFRSF10C	NM_003841	1.543
19	DAXX	NM_001350	0.592	48	PSMC4	NM_006503	0.611
20	CASP10	NM_001230	0.58	49	POLR2J	NM_006234	1.656
21	EPOR	NM_000121	0.668	50	TNFSF10	NM_003810	0.457
22	MAPK1	NM_002745	0.62	51	HSBP1	NM_001537	0.667
23	TNFRSF1A	NM_001065	0.596	52	RIPK2	NM_003821	1.812
24	RGS14	NM_006480	0.425	53	IL2RB	NM_000878	0.707
25	GNA13	NM_006572	3.044	54	SKIL	NM_005414	0.728
26	TP53BP1	NM_005657	0.577	55	CES1	NM_001266	1.855
27	GHSR	NM_004122	2.359	56	DIRAS3	NM_004675	1.765
28	HLTF	NM_003071	0.576	57	AKAP11	NM_016248	0.588
29	CHST4	NM_005769	0.633	58	ZNF354A	NM_005649	0.593

(Satoh et al. Manuscript in preparation)

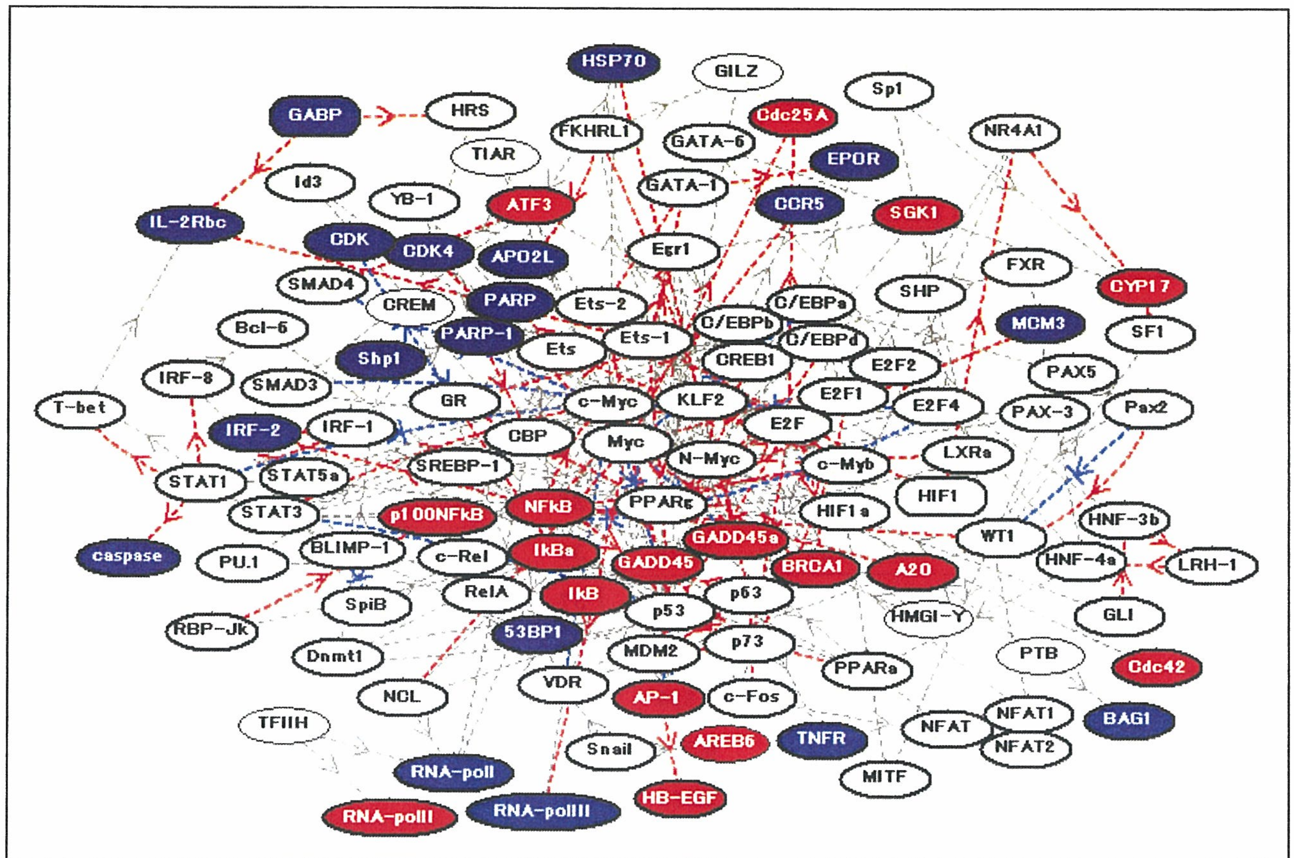

Fig. 2. The common upstream search of 58 differentially expressed genes in T cells between MS subgroup A (MS-A) and normal controls (Nc). The microarray data of 58 differentially expressed genes (MDG) in T cells between MS subgroup A (MS-A) and normal controls (Nc) imported into KeyMolnet extracted 95 genes directly linked to 58 genes. The "common upstream" search of 98 genes generated a molecular network composed of 117 nodes. Red nodes represent upregulated genes in MS-A, whereas purple nodes represent downregulated genes in Nc. White nodes exhibit the genes extracted by KeyMolnet to establish molecular connections. The direction of molecular relation is indicated by dash line with arrow (transcriptional activation) or dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet **(Satoh et al. Manuscript in preparation)**.

(説明文書・同意文書・質問表)

研究協力のお願いにあたって

国立精神・神経センター神経研究所免疫研究部では多発性硬化症（MS）の病態、病因を明らかにするための研究をしております。主旨をよく理解して頂き、御協力していただけましたら有り難く存じます。.

１.MSの関連遺伝子の検索

多発性硬化症（MS）は一つ、二つの遺伝子が決定的な役割を果たす疾患ではありません。同じ遺伝子を持った一卵性双生児の一人 が MS になる確率はおよそ３０％と言われます。従ってある特定の遺伝子の組み合わせをもっている人はMSになりやすい場合があるとはいえ、ある遺伝子を持っているからMSになるのではなく、環境要因が発病に大きく関与することになります。従ってMSにおける遺伝子を調べることによって「ある特定の遺伝子が決定的な役割を持つ」という結論になる可能性は少ないと考えられます。このような場合に自分の遺伝子が明らかになることによって決定的な不利益を被る可能性および、遺伝子が明らかになることによって遺伝子治療などの恩恵を将来受けるようになる可能性は少ないと思われます。しかし、病気になりやすくする遺伝子がはっきりすることによって病気になるしくみを解明する、あるいは新しい治療につながる可能性があります。

２.MSの発症、経過、抑制する細胞の機能の研究

MSはT細胞という白血球の一部が誤って自分の脳を攻撃してしまうために起こると考えられている病気です。どうして患者さんの細胞は自分を攻撃してしまうのか、どうして通常は自分の身体を攻撃しないのかを研究しています。言いかえれば、T細胞が脳を攻撃する機序やそのほかの細胞がどのように T 細胞が脳を攻撃しないように守っているかの研究です。対象となる細胞は血液中のT細胞、NKT細胞、NK細胞や樹状細胞など様々な細胞です。

注意事項

（1） 研究協力の任意性と撤回の自由

この研究への協力の同意はあなたの自由意志で決めてください。強制いたしません。また、同意しなくても、あなたの不利益になるようなことはありません。一旦同意した場合でも、あなたが不利益を受けることなく、いつでも同意を取り消すことができます。また採取した血液や遺伝子を調べた結果を研究に含まないように要求すれば、データは廃棄されます。ただし、同意を取り消した時すでに研究結果が論文などで公表されていた場合などのように、遺伝子を調べた結果などを廃棄することができない場合があります。

（2）研究内容

題目：多発性硬化症の病態に関する研究
研究機関名：国立精神・神経センター神経研究所免疫研究部
研究責任者：山村　隆（部長)、佐藤準一（室長)
目的：多発性硬化症の発症や病気の進行に関与する遺伝子の検索
および　　疾患の病態を細胞、分子レベルで解析
研究方法：血液を通常の方法で 5-30m l 採血します。採血にともなう身体の危険性はほとんどありません。これらの組織に含まれる DNA という物質を取り出し、調べます（研究協力にあたっての１)。調べる対象となる遺伝子については研究の進展によって変わることがあります。または血液中に存在する細胞を分離してその機能を調べます（研究協力にあたっての２)。

（3） 試料提供者にもたらされる利益

研究の成果は今後の医学の発展に寄与します。その結果、将来、あなたの病気の診断や予防より効果的に行われるようになる、さらに新しい治療の発見につながるかもしれません。しかし、その結果がすぐにあなた自身に直接還元できる可能性は低いと考えられます。

（4） 個人情報の保護

研究結果は様々な問題を引き起こす可能性があるため、他の関係する人にもれないように、取り扱いを慎重に行います。

（5） 解析結果の開示

個人個人に対する解析結果を開示することは原則としてありません。偶然に MS 以外の病気が疑われた場合、医療機関を受診することを勧めることはありえます。

（6） 研究成果の公表

あなたの協力によって得られた研究の成果は、提供者本人やその家族の氏名などが明らかにならないようにした上で、学会発表や学術雑誌およびデータベース上等で公に発表されることがあります。

（7） 研究から生じる知的財産権の帰属

研究の結果として特許権などが生じる可能性がありますが、その権利は国、研究機関、民間企業を含む共同研究機関および研究遂行者などに属します。

（8） 研究終了後の試料等の取扱の方針

あなたの血液などの試料は、原則として当研究室での研究のために用いさせていただきます。当研究室に所属する研究者の共同研究者に試料を分与することはありえますが、広く研究用に提供する事業（バンク事業）にあなた（または、あなたが代わりをつとめる提供者本人）からいただいた試料やそれから取り出したＤＮＡなどを提供する予定はありません。。

（9）カウンセリング

あなたが病気のことや遺伝子解析研究に関して、不安に思うことがあったり、相談したいことがある場合は免疫研究部にご連絡ください。ただし、個々の治療方針に関する質問にはお答えできないことがあります。

多発性硬化症研究への協力の同意文書

私は多発性硬化症の遺伝子解析と病態解明に関する研究について説明文書を用いて説明を受け、その方法、危険性、分析結果のお知らせの方法等について十分理解しました。ついては、次の条件で研究協力に同意致します。

説明を受け理解した項目（□の中にご自分で レを付けて下さい。）
□遺伝子の分析を行うこと □細胞の機能解析を行うこと □研究協力の任意性と撤回の自由 □研究目的 □ 研究方法 □試料提供者にもたらされる利益 □個人情報の保護 □遺伝子解析結果の開示 □研究成果の公表 □研究から生じる知的財産権の帰属

私は私が提供する生体試料等が遺伝子解析研究に使用されることに同意します。
はい　　いいえ

私は私が提供する生体試料等が細胞機能研究に使用されることに同意します。
はい　　いいえ

平成　　年　月　日
署名（活字体）：

年齢
住所
電話

説明者

国立精神・神経センター
神経研究所免疫研究部

質問表

氏名

年令　　性

多発性硬化症（MS）には様々な経過や症状があります。また治療が結果に関係することがあります。以下の質問に答えられる部分だけ（答えたくなければ空欄でけっこうです）、お答えください。

1．最初にMSの症状が出現したのはいつですか。

（　　　　　　　　　　　　　　）

2．それはどんな症状でしたか。

（　　　　　　　　　　　　　　　　　　　　　　　　）

3．MSと診断されたのはいつ、どこでですか。

（　　　　　　　　　　　　　　　　　　　　　　　　）

4．症状は落ち着いていて進行しない時期、改善する時期があってまたある時に新しい症状が出たり悪くなったりする方は1に、最初の症状が出現してからは症状が停止した時期のない方は2に、最初は良くなったり悪くなったりしていたが現在は症状が進行性である方は3に○をつけてください。

1　2　3

5．現在、ある症状について教えてください。

（

）

6．現在、症状は進行していますか。停止していますか。また最後に再発があったのはいつですか。

（　　　　　　　　　　　　　　　　　　　　　　　　）

7．現在、ステロイド、免疫抑制剤、インターフェロンなどで治療されている方は教えてください。パルス療法を3ヶ月以内にされた方はいつされたか、教えてください。ない方はないとお答えください。

（　　　　　　　　　　）

本日はありがとうございました。要望などございましたらお知らせください。

（　　　　　　　　　　　　　　　　　　　　　　　　）

III. 研究成果の刊行に関する一覧表

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
Satoh J	Protein Microarray Analysis for Rapid Identification of 14-3-3 Protein Binding Partners.	Predki PF	Functional Protein Microarrays in Drug Discovery.	CRC Press	Boca Raton, FL	2007	in press (total 21 pages)

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
Satoh J, Nakanishi M, Koike F, Miyake S, Yamamoto T, Kawai M, KikuchiS, NomuraK, Yokoyama K, Ota K, KandaT, FukazawaT, YamamuraT	Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis.	Neurobiology of Disease	18 (3)	537-550	2005
Satoh J, Onoue H, Arima K, Yamamura T	Nogo-A and Nogo receptor expression in demyelinating lesions of multiple sclerosis.	Journal of Neuropathology and Experimental Neurology	64 (2)	129-138	2005
Satoh J, Onoue H, Arima K, Yamamura T	The 14-3-3 protein forms a molecular complex with heat shock protein Hsp60 and cellular prion protein.	Journal of Neuropathology and Experimental Neurology	64 (10)	858-868	2005
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Chiba A, Kaieda S, Oki S, Yamamura T, Miyake S	The involvement of Va14 natural killer cells in the pathogenesis of arthritis in murine models.	Arthritis and Rheumatism	52 (6)	1941-1948	2005
Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M, Yamamura T, Chayama K	Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice.	Inflammatory Bowel Diseases	11 (1)	35-41	2005
Murata K, Toba T, Nakanishi K, Takahashi B, Yamamura T, Miyake S, Annoura H.	Total synthesis of an immunosuppressive glycolipid, (*2S,3S,4R*)-1-O- (α-D-galactosyl)-2- tetracosanoylamino-1,3,4-nonanetriol.	Journal of Organic Chemistry	70 (6)	2398-2401	2005
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南里悠介、佐藤準一、佐藤和貴郎、山村隆	DNAマイクロアレイによる多発性硬化症の診断とインターフェロンベータ治療反応性予測に関するアンケート調査.	神経内科	64 (3)	319-320	2006
佐藤準一	多発性硬化症のマイクロアレイ診断. 特集II 多発性硬化症研究・治療の現状2006.	神経研究の進歩	50 (4)	582-599	2006
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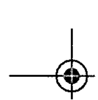
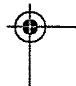
Satoh J, Nanri Y, Tabunoki H, Yamamura T	Microarray analysis identifies a set of CXCR3 and CCR2 ligand chemokines as early IFNβ-responsive genes in peripheral blood lymphocytes: an implication for IFNβ-related adverse effects in multiple sclerosis.	BMC Neurology	6 (May 19)	18-34	2006
Satoh J, Tabunoki H, Nanri Y, Arima K, Yamamura T	Human astrocytes express 14-3-3 sigma in response to oxidative and DNA-damaging stresses.	Neuroscience Research	56 (1)	61-72	2006
Aranami T, Miyake S, Yamamura T	Differential expression of CD11c by peripheral blood NK cells reflects temporal activity of multiple sclerosis.	Journal of Immunology	177 (8)	5659-5667	2006
Croxford JL, Miyake S, Huang YY, Shimamura M, Yamamura T	Invariant Vα 19i T cells regulate autoimmune inflammation.	Nature Immunology	7 (9)	987-994	2006
Miyamoto K, Miyake S, Mizuno M, Oka N, Kusunoki S, Yamamura T	Selective COX-2 inihibitor celecoxib prevents experimental autoimmune encephalomyelitis through COX-2-independent pathway.	Brain	129 (Pt 8)	1984-1992	2006
PyZ E, Naidenko O, Miyake S, Yamamura T, Berberich I, Cardell S, Kronenberg M, Herrmann T	The complementary determinating region 2 of BV8S2 (V beta 8.2) contributes to antigen recognition by rat invariant NKT cell TCR.	Journal of Immunology	176 (12)	7447-7455	2006

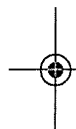
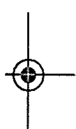
IV. 研究成果の刊行物・別刷

13 Studying Protein–Protein Interactions with Protein Microarrays: Rapid Identification of 14-3-3 Protein Binding Partners

Jun-ichi Satoh

CONTENTS



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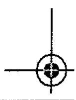 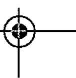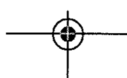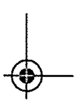 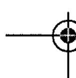

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INTRODUCTION

The 14-3-3 Protein Acts as a Molecular Adaptor in Signaling Networks

The 14-3-3 protein family in mammalian cells consists of evolutionarily conserved, acidic 30-kDa proteins composed of seven isoforms named β, γ, ε, ζ, η, θ, and σ.[1,2] A homodimeric or heterodimeric complex composed of the same or distinct isoforms constitutes a large cup-like structure possessing an amphipathic groove with two ligand-binding capacity, and acts as a molecular adaptor by interacting with key signaling components of cell differentiation, proliferation, transformation, and apoptosis. The dimeric 14-3-3 protein regulates the function of target proteins by restricting their subcellular location, bridging them to modulate catalytic activity, and protecting them from dephosphorylation or proteolysis.[3,4] Although 14-3-3 is widely distributed in neural and nonneural tissues, it is expressed at the highest level in neurons in the central nervous system (CNS).[5,6] Aberrant expression and impaired function of 14-3-3 in the CNS are closely associated with pathogenetic mechanisms of various neurological disorders, such as Creutzfeldt-Jacob disease,[7–9] Alzheimer disease,[10] Pick disease,[11] Parkinson disease,[12,13] multiple system atrophy,[14,15] spinocerebellar ataxia,[16] amyotrophic lateral sclerosis,[17] Miller-Diecker syndrome,[18] multiple sclerosis,[19,20] and mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS).[21,22]

In general, the 14-3-3 protein interacts with phosphoserine-containing motifs of its ligands, such as RSXpSXP (mode I), RXXXpSXP (mode II), and pS/pT(X_{1-2})COOH (mode III), in a sequence-specific manner.[23,24] Until present, more than 300 proteins have been identified as being 14-3-3-binding partners. They include Raf-1 kinase, Bcl-2 antagonist of cell death (BAD), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and cdc25 phosphatase.[1,2,25] Binding of 14-3-3 to Raf-1 is indispensable for its kinase activity in the Ras-MAPK signaling pathway, while the interaction of 14-3-3 with BAD, when phosphorylated by a serine/threonine kinase Akt, inhibits apoptosis. Furthermore, recent studies indicate that the 14-3-3 protein may also interact with a set of target proteins in a phosphorylation-independent manner.[26–29] Increasing our knowledge of molecular interactions between 14-3-3 and target proteins would greatly help us to understand the biological function and pathological implication of the 14-3-3 protein networks.

The Advantages of Protein Microarray Analysis to Identify Protein–Protein Interactions

The yeast two-hybrid (Y2H) system is a powerful approach to identify novel protein–protein interactions in a high-throughput fashion.[30,31] However, Y2H screening requires a lot of time and effort, and is often criticized for detecting the interactions

unrelated to the physiological setting and obtaining high rates of false positive interactors caused by spontaneous activation of reporter genes and self-activating bait proteins.[32,33] Affinity purification coupled with mass spectrometry (APMS) is an alternative approach to identify the components of protein complexes on a large scale. This approach has been taken to identify a wide range of 14-3-3-interacting proteins involved in the dynamic control of cytoskeletons,[34] cell cycle regulation,[35] biosynthetic metabolism,[36] and oncogenic signaling events.[37] Although APMS screening detects binding partners of physiological significance, it is also time-consuming and expensive, requires a large amount of samples, and has a difficulty in detecting transmembrane proteins and loosely associated components that might be lost during purification.[38] Furthermore, the recognized interaction is not always direct, assisted by intermediary molecules.

Recently, protein microarray technology has been established for the rapid, systematic, and less expensive screening methods of thousands of protein–protein, protein–lipid, and protein–nucleic acid interactions in a high-throughput fashion.[39–43] It requires small sample volumes and affords the ability to control the experimental parameters, such as buffer pH, ion concentration, and reaction cofactors in a reproducible manner. This approach has diverse applications to discovery-based proteomics in the field not only of basic biological research but also of drug and biomarker discovery research, including identification of the substrates of protein kinases, the protein targets of small molecules, the consensus interaction of transcription factors, and autoantibody profiling.[44–51] Thus, this technology sounds pivotal for establishment of personalized medicine. The vast majority of protein–protein interactions occur between a domain located in one protein and a small motif spanning usually 8 to 15 amino acids in its ligand. They promote multimolecular protein complex formation that regulates diverse signaling networks. A recent study using the microarray containing 212 spots of protein domains, composed of two conserved tryptophans (WW), two conserved phenylalanines (FF), Src homology 2 (SH2), Src holmology 3 (SH3), pleckstrin homology (PH), forkhead-associated (FHA), PSD-95, DLG and ZO-1 proteins (PDZ), and 14-3-3-interacting modules, characterized the domain-specific binding profile of various signaling molecules in a single experiment.[52] More recently, the epidermal growth factor receptor (EGFR) signaling network was studied by using protein microarrays that contain virtually all SH2 and phosphotyrosine binding (PTB) domains encoded in the human genome, and probing with phosphotyrosine (pY)-containing peptides derived from EGFR, ErbB2, and ErbB3.[53]

Here, we have attempted to characterize a comprehensive human 14-3-3 interactome by analyzing a high-density protein microarray.

EXPERIMENTAL PROTOCOLS

Preparation of an Epitope-Tagged Probe for Microarray Analysis

Human embryonic kidney cells HEK293 whose genome was modified for the Flp-In system (Flp-In 293) were obtained from Invitrogen, Carlsbad, CA. Flp-In 293 cells contain a single Flp recombination target (FRT) site targeted for the site-specific

recombination, integrated in a transcriptionally active locus of the genome, where it stably expresses the *lacZ*-Zeocin fusion gene driven from the pFRT/*lac*Zeo plasmid under the control of SV40 early promoter. Flp-In 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (feeding medium) with inclusion of 100 μg/ml of Zeocin (Invitrogen) according to the methods described previously.[54]

To prepare the probe for protein microarray analysis, the open reading frame (ORF) of the human 14-3-3ε gene (YWHAE, GenBank accession No. NM_006761, amino acid residues 2 to 255) was amplified from cDNA of NTera 2-N cells, a model of differentiated human neurons in culture [55], by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the sense (5'gatgatcgagaggatctggtgtac3') and antisense (5'ctgattttcgtcttccacgtcctg3') primers. The PCR product was then cloned into a mammalian expression vector pSecTag/FRT/V5-His TOPO (Invitrogen) to produce a fusion protein with a C-terminal V5 (GKPIPNPLLGLDST) tag, a C-terminal polyhistidine (6xHis) tag, and an N-terminal Ig κ-chain secretion signal. This vector, together with the Flp recombinase expression vector pOG44 (Invitrogen), was transfected in Flp-In 293 cells by Lipofectamine 2000 reagent (Invitrogen) (Figure 13.1). A stable cell line was established after incubating the cells for approximately one month in the feeding medium with inclusion of 100 μg/ml of Hygromycin B (Invitrogen). The stable cell line was named 293eV5.[56] In this system, the recombinant protein was secreted into the culture medium after the Ig κ-chain secretion signal sequence was processed by an endogenous signal peptidase-mediated cleavage. Therefore, it has an advantage of easily purifying the recombinant protein, compared with the system where the recombinant protein is expressed in the cytoplasm, mixed with various unnecessary proteins.

To purify the recombinant 14-3-3ε protein, the culture supernatant of 293eV5 incubated in the serum-free DMEM/F-12 medium for 48 hours was harvested and concentrated at an 1/40 volume by centrifugation on an Amicon Ultra-15 filter (Millipore, Bedford, MA). It was then purified by the HIS-select spin column (Sigma, St. Louis, MO), and concentrated at a 1/10 volume by centrifugation on a Centricon-10 filter (Millipore). The purity and specificity of the probe were verified by Western blot analysis using mouse monoclonal anti-V5 antibody (Invitrogen) and rabbit polyclonal antibody specific for the 14-3-3ε isoform (IBL, Gumma, Japan) (Figure 13.1).

PROTEIN MICROARRAY ANALYSIS

ProtoArray human protein microarray (v1.0; Invitrogen) we utilized contains 1752 human proteins of various functional classes spotted in duplicate on a nitrocellulose-coated glass slide. (After a quality control procedure, the number of total arrayed proteins is reduced from 1900 originally listed in the array.) Nitrocellulose-coated surface provides a nearly quantitative retention of the spotted proteins and significantly higher detection sensitivity than the other surfaces.[40] All the proteins immobilized on the array were expressed as an N-terminal glutathione-S transferase (GST)-6xHis fusion protein derived from the genes selected from the human

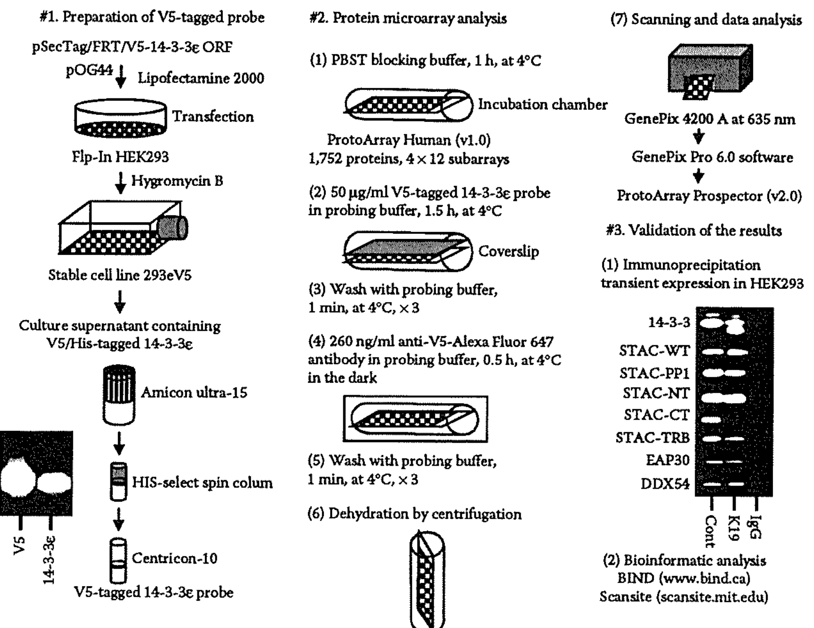

FIGURE 13.1 Protein microarray analysis of 14-3-3-binding proteins. The experimental protocol is comprised of the following three steps. (1) Preparation of V5-tagged probe. The recombinant human 14-3-3ε protein tagged with V5 was purified from the concentrated culture supernatant of a stable cell line 293eV5. The purity and specificity of the probe were verified by Western blot analysis using anti-V5 antibody or anti-14-3-3ε antibody. (2) Protein microarray analysis. After blocking nonspecific binding, the microarray containing 1752 human proteins was incubated with the probe, followed by incubation with anti-V5 antibody labeled with Alexa Fluor 647, and scanned on the microarray scanner. The significant binding was identified by analyzing the data with the ProtoArray Prospector software. (3) Validation of the results. The specific binding to 14-3-3 of STAC (WT, wild type; PP1, WT with inclusion of protein phosphatase-1 during protein extraction; NT, the N-terminal half; CT, the C-terminal half; TRB, the truncated form lacking both the RYYSSP motif and the cysteine-rich domain), EAP30 and DDX54 was validated by immunoprecipitation (IP) analysis of the corresponding recombinant proteins expressed in HEK293 cells. Lanes represent the input control (Cont), and IP with K-19 or normal rabbit IgG. The 14-3-3 interactors identified by protein microarray analysis were further evaluated by bioinformatic analysis of protein–protein interaction networks via BIND and Scansite database searches.

ultimate ORF clone collection (Invitrogen). They represent either the full-length or the partial fragment of recombinant proteins. They were expressed in Sf9 insect cells by using the Bac-to-Bac Baculovirus expression system (Invitrogen), purified under non-denaturating conditions by glutathione affinity chromatography in the presence of protease inhibitors, and processed for spotting on the slides (Invitrogen application note).

The proteins are spotted in an arrangement composed of 4 × 12 subarrays equally spaced in vertical and horizontal directions. Each subarray includes 16 × 16 spots, composed of 48 control spots (C), 80 human proteins (P), and 128 blanks (B) (Figure 13.2a). The controls include 14 positive control spots; four spots of an Alexa Fluor 647-labeled antibody (rows 1, 8; columns 1, 2), six spots of a concentration gradient of a biotinylated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (row 8; columns 3 to 8), and four spots of a concentration gradient of V5 protein (row 8; columns 13 to 16). They also include 34 negative control spots; six spots of a concentration gradient of bovine serum albumin (BSA) (row 1; columns 3 to 8), four spots of a concentration gradient of a rabbit anti-GST antibody (row 1; columns 9 to 12), four spots of a concentration gradient of calmodulin (row 1; columns 13 to 16), 16 spots of a concentration gradient of GST (row 2; columns 1 to 16), two spots of buffer only (row 8; columns 9,10), and two spots of an anti-biotin antibody (row 8; columns 11, 12).

Nonspecific binding was blocked by incubating the microarray for 60 min at 4in the PBST blocking buffer composed of 1% BSA and 0.1% Tween 20 in phosphate-buffered saline (PBS), as described previously (Figure 13.1).[56] Then, it was incubated for 90 min at 4 with the probe described above at a concentration of 50 μg/ml in the probing buffer composed of 1% BSA, 5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.05% Triton X-100, and 5% glycerol in PBS. The array was washed three times with the probing buffer, followed by incubation for 30 min at 4with mouse monoclonal anti-V5 antibody conjugated with Alexa Fluor 647 (Invitrogen) at a concentration of 260 ng/ml in the probing buffer. The array was washed three times with the probing buffer, dehydrated by brief centrifugation, and then scanned by the GenePix 4200A scanner (Axon Instruments, Union City, CA) at a wavelength of 635 nm. The data in a format specified by the GenePix Pro 6.0 microarray data acquisition software (Axon Instruments) were analyzed by using the ProtoArray Prospector software v2.0 (Invitrogen) following acquisition of the microarray lot-specific information online (www.invitrogen.com/protoarray). The spots showing the background-subtracted signal intensity value greater than the median plus three standard deviations of intensities of all protein features were considered as having a significant binding.

The Z-Score was calculated by the following formula: $Z_k = (X_k - \mu_s)/\sigma_s$, where X_k represents the signal intensity value of the *k*th protein feature, μ_s is the mean signal intensity of all protein features, and σ_s expresses the standard deviation of intensities of all protein features. The Z-Score reflects a binding specificity determined by the definition how far and in what direction a signal from a specific protein feature deviates from the mean signal intensity of all the protein features.

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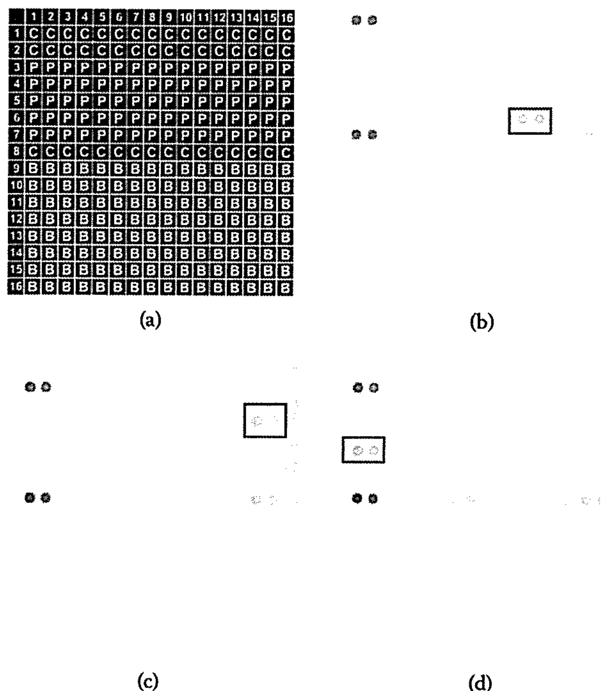

FIGURE 13.2 Detection of 14-3-3-binding proteins on protein microarray. The microarray we utilized contains 1752 distinct human proteins of various functional classes spotted in duplicate on a nitrocellulose-coated glass slide. They are printed in an arrangement of 4 × 12 subarrays equally spaced in vertical and horizontal directions. (a) Layout of the subarray. Each subarray includes 16 × 16 spots composed of 48 control spots (C), 80 human proteins (P), and 128 blanks (B). (b) EAP30 on the subarray 1. The spots of (row 7; column 1) and (row 7; column 12) indicated by a square represent EAP30. (c) DDX54 on the subarray 27. The spots of (row 3; column 15) and (row 3; column 16) indicated by a square represent DDX54. (d) STAC on the subarray 39. The spots of (row 5; column 1) and (row 5; column 2) indicated by a square represent STAC. In these subarrays (b–d), the positive control spots represent an Alexa Fluor 647-labeled antibody (rows 1, 8; columns 1, 2) that provides the strong signals, a concentration gradient of a biotinylated anti-mouse antibody with a capacity to bind to mouse monoclonal anti-V5 antibody labeled with Alexa Fluor 647 (row 8; columns 3 to 8), and a concentration gradient of V5 protein (row 8; columns 13 to 16). The signals are only visible at the higher concentration in the latter two.

VALIDATION AND EVALUATION OF THE RESULTS OF PROTEIN MICROARRAY ANALYSIS

Transient Expression of Recombinant Proteins in HEK293 Cells

To verify the results of protein microarray analysis, the ORF of the genes encoding EAP30 subunit of ELL complex (EAP30, NM_007241, amino acid residues 2 to 258), dead box polypeptide 54 (DDX54, NM_024072, amino acid residues 2 to 881), and

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src homology three (SH3) and cysteine rich domain (STAC, NM_003149, amino acid residues 2 to 402, full-length) were amplified by PCR using PfuTurbo DNA polymerase and the specific primer sets (5'caccgccgcggggtgggagctggc3' and 5'tcaggggagggcttctctg-gcctc 3' for EAP30; 5'gcggccgacaagggcccggcggct3' and 5'tcacatcctcttccgcatcttgcc3' for DDX54; and 5'atccctccgagcagcccccgcgag3' and 5'tcagatgttttctagtacatcaag3' for STAC). The N-terminal half of STAC (amino acid residues 2 to 333, NTF), the C-terminal half of STAC (amino acid residues 234 to 402, CTF), and two distinct truncated forms of STAC (amino acid residues 2 to 164 named TRA and amino acid residues 2 to 105 named TRB) were amplified using the corresponding primer sets (5'atccctccgagcagcccccgcgag3' and 5'tcaaagatctgaagtagaggttct3' for NTF; 5'gtggaggt-tcctgaggaagccaat 3' and 5'tcagccacctggatgcagaccagc3' for CTF; 5'atccctccgagcagc-ccccgcgag 3' and 5'tcatggcagcttgcccatgcaccg3' for TRA; and 5'atccctccgagcag- ccccc gcgag 3' and 5'tcagccacctggatgcagaccagc3' for TRB).

They were then cloned into a mammalian expression vector pcDNA4/HisMax-TOPO (Invitrogen) to produce a fusion protein with an N-terminal Xpress tag. To express the STAC mutant with a single amino acid substitution S172A (the single mutant; SMT) or with double amino acid substitutions S172A and S173A (the double mutant; DMT), the pcDNA4/HisMax-TOPO vector containing the full-length wild-type (WT) STAC gene was modified by consecutive site-directed mutagenesis using QuikChange II site-directed mutagenesis kit (Stratagene) and the primer sets (5'gtttcggcgttactacgcctccccttgctcattc3' and 5'gaatgagcaaggggggaggcgtagtaacgccgaaac 3' for SMT and 5'cggcgttactacgccgccccttgctcattcat3' and 5'atgaatgagcaagggggcggc gtagtaacgccg3' for DMT). All these vectors were transfected in HEK293 cells by Lipofectamine 2000 reagent.

Coimmunoprecipitation Analysis

For coimmunoprecipitation analysis, total protein extract was prepared by homogenizing the cells in M-PER lysis buffer (Pierce, Rockford, IL) supplemented with a cocktail of protease inhibitors (Sigma), either with inclusion of phosphatase inhibitors (Sigma) to maintain the protein phosphorylation status or with inclusion of recombinant protein phosphatase-1 (PP1) catalytic subunit α-isoform (5 U/ml; Sigma) instead of phosphatase inhibitors to induce the protein dephosphorylation reaction.[57] The homogenate was centrifuged at 12,000 rpm for 20 min at 4. After preclearance, the supernatant was incubated for 3 hours at 4 with 30 μg/ml rabbit polyclonal anti-14-3-3 protein antibody (K19)-conjugated agarose (Santa Cruz Biotechnology, Santa Cruz, CA) or the same amount of normal rabbit IgG-conjugated agarose (Santa Cruz Biotechnology). After several washes, the immunoprecipitates were processed for Western blot analysis using mouse monoclonal anti-14-3-3 protein antibody (H-8, Santa Cruz Biotechnology) and mouse monoclonal anti-Xpress antibody (Invitrogen). K-19 and H-8 antibodies recognize all 14-3-3 isoforms. The specific reaction was visualized by using a chemiluminescent substrate (Pierce).

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Bioinformatic Analysis

In addition to validation of the specific interactions by wet experiments, we evaluated them by bioinformatic analysis. The information on known 14-3-3 interactors, molecular