予知出来れば、早期治療を開始して軽症化出来る。治療が遅れれば永久的後遺症を残す。しかし現在までMS再発予測法は確立されていない。MSは通常の血液検査では異常を認めず、神経学的所見・経過・脳脊髄液所見・MRI画像に基づいて診断されるが、鑑別疾患が多く、神経内科専門医でも正確な診断は容易ではない。MS病態解明のためには、発症を正に制御すると考えられる自己反応性T細胞を含む細胞集団を選択的に濃縮して解析する必要がある。

本研究では多数のMS症例の末梢血Tリンパ球遺伝子発現プロフィールを遺伝子アレイで網羅的に解析、MSの免疫病態の解明・診断法の開発を目指した。分担研究者山村は主として検体収集を担当した。

B. 研究方法

文書で同意を取得した国立精神・神経センター武蔵病院神経内科通院・入院中のMS患者とnon-MS対照神経疾患患者、および健常者ボランティア(normal control subjects; Nc)から、Ficoll-Paque遠心分離法で末梢血リンパ球(peripheral blood mononuclear cells; PBMC)を採取、MACSで$CD3^+$ T細胞を分離、RNAを精製、遺伝子アレイ(cDNA microarray, 1258 genes; Hitachi Life Science)を用いて、遺伝子発現プロフィールを解析した。またIlles准教授(Hungary Pecs大学)との共同研究で、Hungary人MS 33検体(4 MS twin pairsを含む)とNc 22検体を収集した(航空便で輸送)。全サンプルを-80℃で凍結保存した。以下に示すプロジェクト(佐藤・山村)を施行した。

(1)Relapsing-remitting MS(RRMS)患者(n = 6)で、神経内科専門医の診断に基づく急性増悪期(acute relapse)と完全寛解期(complete remission)に$CD3^+$ T細胞を採取、DNAマイクロアレイで両者を比較し再発期特異的遺伝子群を同定した(佐藤総括報告書参照)。

(2)Hungarian monozygotic (MZ) twin 4 pairs (#1. MS/MS; #2. MS/MS; #3. MS/MS; #4. MS/Nc)の$CD3^+$ T細胞をDNAマイクロアレイで比較解析し、MS特異的に発現亢進している遺伝子群を抽出、KeyMolnet(医薬分子設計研究所 IMMD)を用いて、共通上流検索法(発現制御に関与する転写因子群を調べる)で分子ネットワークを解析した。

(3)平成17年度の本研究で樹立したMS病型分類データベース(MS classification database; MSCD)を用いて、新規91例(MS = 78; non-MS = 8; Nc = 5)に関して、階層的クラスター解析(hierarchical clustering analysis; HCA)とサポートベクターマシン解析(support vector machine; SVM)に基づくMS診断法を開発し、その精度を検証した。SVMではMS群とNc群の識別関数(Kernel function)として、polynomial dot product (PDP)- 1, 2, 3とradial basis (Gaussian)を用いた。

(倫理面への配慮)

「多発性硬化症患者および対照リンパ球遺伝子発現解析研究(申請者山村隆)」は、既に国立精神・神経センター倫理委員会で承認済みである。本研究で解析する患者全員から研究参加に関して文書で同意を取得した。また全サン

プルは第3者により暗号化し、検査者には個人情報がわからないようにし取り扱うように配慮した。

C. 研究結果

#1. MS 再発期特異的遺伝子(relapse-specific genes; RSG)の解析：平成18年度総括報告書(佐藤)を参照。(Satoh et al. Submitted for publication).

#2. Hungarian MZ twin 解析 : 4 pairs(concordant pairs; #1. MS/MS; #2. MS/MS; #3. MS/MS; discordant pair: #4. MS/Nc)の $CD3^+$ T 細胞を遺伝子アレイで解析、concordant pairs(#1-3)で各々発現差異を認める上位 100 遺伝子(100 DEG)を抽出した。次に discordant pair (#4)で発現差異を認める上位 50 遺伝子(50 DEG)を抽出した。最終的に 50 DEG 中で 100 DEG に含まれない 34 遺伝子から、Nc に比較し MS で2倍以上の発現差異を呈する 20 遺伝子(20 DEG)を抽出、MS-specific genes (MSG)と命名した(Table 1)。20 DEG について、KeyMolnet で関連 44 遺伝子を抽出、共通上流検索法で分子ネットワークを解析した。その結果、MS 特異的遺伝子発現プロフィールの特徴として、NF-κB および Ets 転写因子ファミリーを介する発現制御系の関与が示唆された(Fig. 1)(Satoh et al. Submitted for publication)。

#3. HCA, SVM に基づく MS 診断法：MSCD による MS 分類で、最も Nc に近い発現プロフィールを呈した MS サブグループである A 群に注目し、MS-A と NC の群間検定で差異を認めた 58 遺伝子(MS diagnostic genes; MDG)を抽出した(Table 2)。58 MDG について、KeyMolnet で関連 95 遺伝子を抽出、共通上流検索法で分子ネットワークを解析した。その結果 MS と Nc を識別する遺伝子の発現において Myc/Mad および p63 を介する発現制御系の関与が示唆された(Fig. 2)(Manuscript in preparation)。58 MDG を指標とする HCA 解析および MSCD を training set として機械学習させた SVM 解析では、診断正答率(MS を MS と診断し non-MS/Nc を Nc と診断)は HCA 29.7%, SVM 33.0%であった。擬陰性率(MS を Nc と誤診断)は HCA 70.5%, SVM 70.5%で、擬陽性率(Nc/non-MS を MS と誤診断)は HCA 69.2%, SVM 46.2%であった。すなわち 58 MDG を指標とする HCA 識別法単独または SVM 識別法単独では診断正答率が低く、誤診率が高いことが判明した。

D. 考察

非常に貴重なハンガリー人一卵性双生児4ペアの末梢血 $CD3^+$ T 細胞収集し、DNA マイクロアレイで比較解析、MS 特異的遺伝子(20 DEG)の発現プロフィールを明らかにした。その特徴として NF-κB および Ets 転写因子ファミリーを介する発現制御系を同定した。NF-κB は TNFα, IL-1 など炎症性サイトカインの発現制御において中心的役割を果たす転写因子である。また Ets 転写因子ファミリーは T 細胞分化を制御し、自己免疫の発症に関与する。

DNA マイクロアレイ解析では多数のデータセットの要素特性を階層的クラスター解析(HCA)で分類する。教師なし法(unsupervised

method)ではサンプルに関する事前情報なしに、類似発現パターンを呈するサンプルをクラスター(グループ)に分類し、クラスターを識別し得る指標遺伝子(discriminator genes)を抽出する。サポートベクターマシン(support vector machine; SVM)は、指標遺伝子抽出に用いたデータを training set として機械学習し、新規データセットにおけるクラスターを識別可能な超平面(hyperplane)を同定する解析方法(教師あり法 supervised method)である。

また平成 17 年度の本研究で樹立した MSCD を利用して、末梢血 T リンパ球 DNA マイクロアレイ解析 HCA, SVM に基づく MS 診断法の樹立を試みた。非常に残念なことに、指標遺伝子 58 MDG による HCA or SVM 単独では擬陰性率(false negative rate)が極めて高く、MS と non-MS/Nc を識別困難であることが判明した。臨床情報(時間的・空間的な多発性)との組み合わせにより、さらに診断精度を向上出来るか、今後再解析する予定である。

E. 結論

Hungarian MZ twin 解析により、MS 免疫病態で中心的役割を果たす転写因子系ネットワークを解明出来た(Satoh et al. Submitted for publication)。

F. 健康危険情報

総括報告書(佐藤)を参照。

G. 研究発表

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H. 知的所有権の取得状況

1. 特許取得

1) 新規な糖脂質及びこれを有効成分とする自己免疫疾患治療薬(国際公開番号:WO2003/016326).
2) 多発性硬化症に対するインターフェロン・ベータ薬物治療の有効性予測法(特開2004-28926).
3) 多発性硬化症に関連する遺伝子の発現測定方法、多発性硬化症関連遺伝子の発現を測定するためのチップ、多発性硬化症の罹患を判断するための遺伝子群、多発性硬化症の評価方法. (特開2005-160440).
4) 多発性硬化症再発予測法(特許申請中).

2. 実用新案登録
なし

3. その他
なし

Table 1. Twenty Differentially Expressed Genes in T Cells between MS and Nc of Hungary MZ Twin Pair #4 (MS-Specific Genes; MSG)

Gene Symbol	GenBank Accession Number (RefSeq)	MS vs NC Ratio
CYP2D6	NM_000106	3.18
IL11RA	NM_004512	2.6
MAPKAPK2	NM_004759	0.41
AKAP10	NM_007202	2.46
USP6	NM_004505	2.36
COMT	NM_000754	0.44
MAPK3	NM_002746	0.44
SLC6A2	NM_001043	2.28
IL3RA	NM_002183	2.26
SELPLG	NM_003006	0.46
IFNA5	NM_002169	2.18
PTPN7	NM_002832	0.46
NR1H2	NM_007121	0.46
NR2C2	NM_003298	2.11
TGFA	NM_003236	2.08
ITGB1	NM_002211	0.48
ATP6V0D1	NM_004691	0.48
CCL3	NM_002983	2.06
CXCL1	NM_001511	2.04
TNFRSF9	NM_001561	2

(Satoh et al. Submitted for publication)

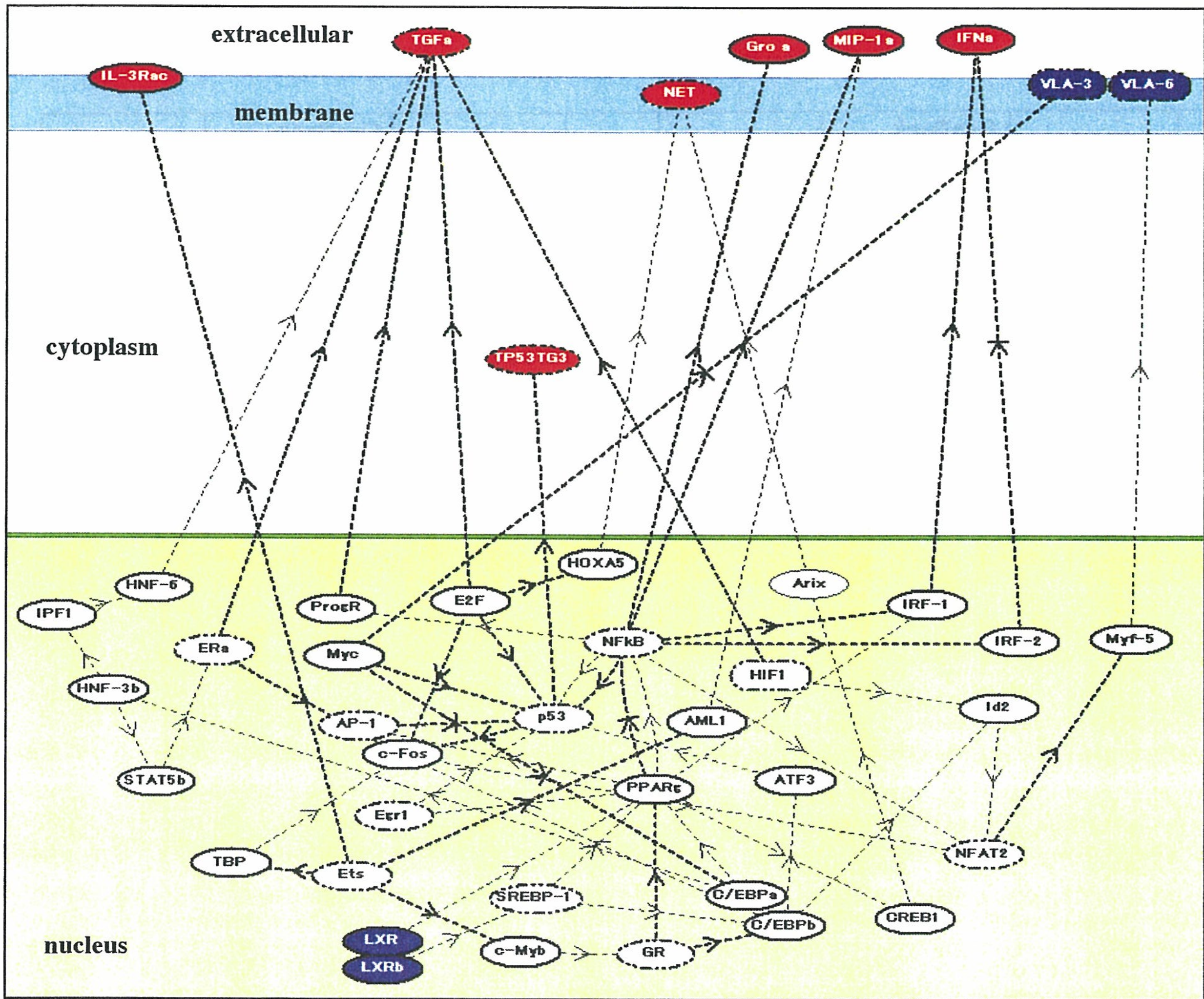

Fig. 1. The common upstream search of 20 differentially expressed genes in T cells between the discordant MS twin. The microarray data of 20 DEG in T cells between the discordant MS twin imported into KeyMolnet extracted 44 genes directly linked to 20 DEG. The "common upstream" search of 44 genes generated 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)

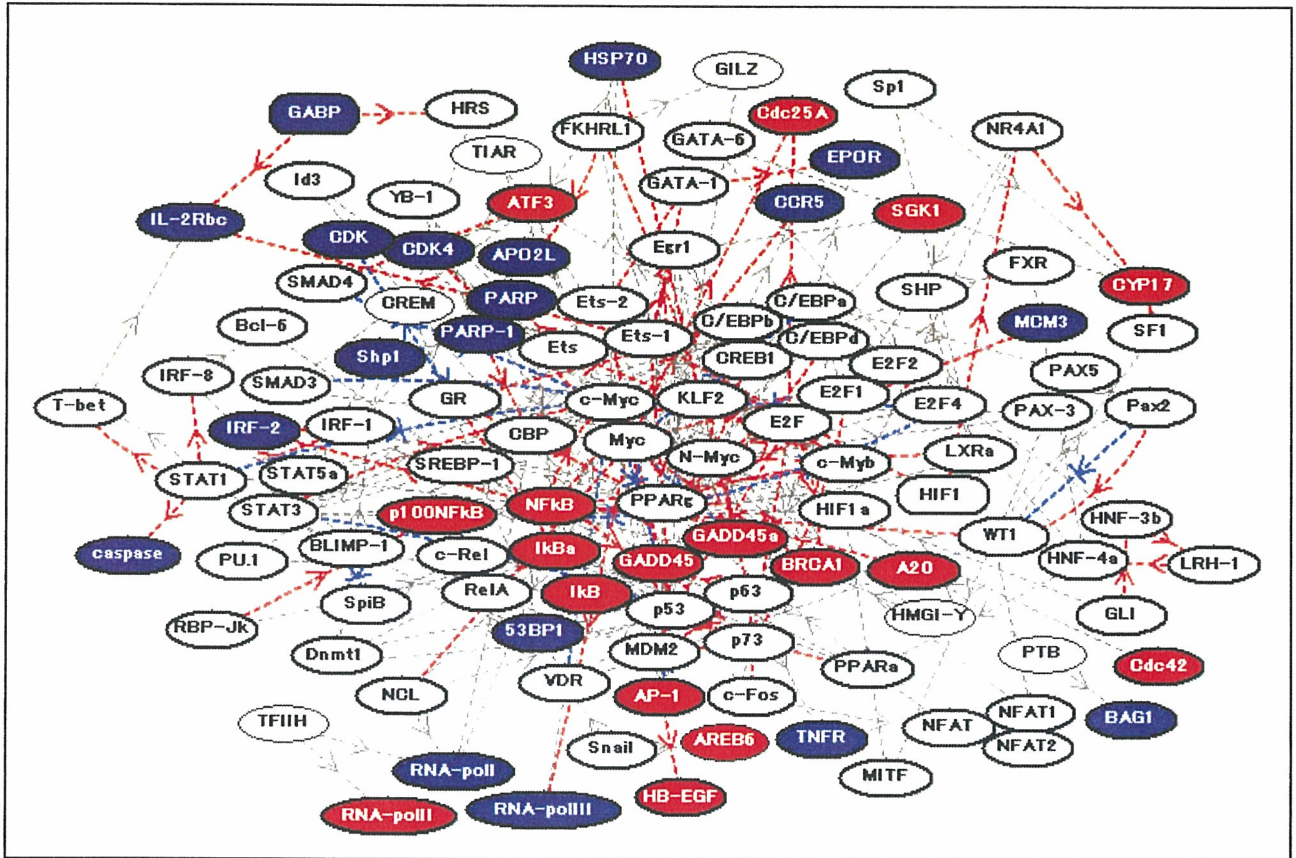

Fig. 2. The common upstream search of 58 differentially expressed genes in T cells between MS subgroup A (MS-A) and normal controls (Nc). The microarray data of 58 differentially expressed genes (MDG) in T cells between MS subgroup A (MS-A) and normal controls (Nc) imported into KeyMolnet extracted 95 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).**

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
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Satoh J, Nakanishi M, Koike F, Onoue H, Aranami T, Yamamoto T, Kawai M, Kikuchi S, Nomura K, Yokoyama K, Ota K, Saito T, Ohta M, Miyake S, Kanda T, Fukazawa T, Yamamura T	T cell gene expression profiling identifies distinct subgroups of Japanese multiple sclerosis patients.	Journal of Neuroimmunology	174 (1-2)	108-118	2006
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
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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

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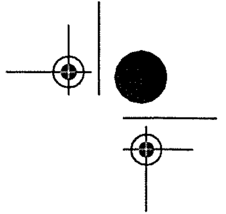
Satoh J, Tabunoki H, Yamamura T, Arima K, Konno H.	Human astrocytes express aquaporin-1 and aquaporin-4 in vitro and in vivo.	Neuropathology		in press	2007

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

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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($X_{1\text{-}2}$)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

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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