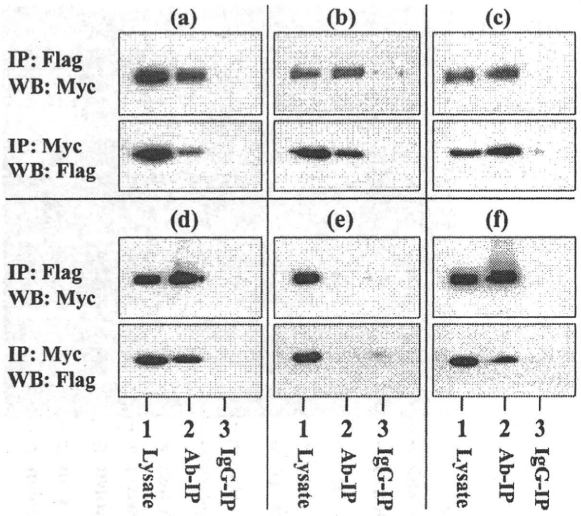


Figure 3. Coimmunoprecipitation analysis. PR209, the N-terminal (NT) half, and the C-terminal (CT) half were expressed as a Flag-tagged fusion protein, while FAM64A, HOXA1, PLK3 and MPG were expressed as a Myc-tagged fusion protein in HEK293 cells. Immunoprecipitation (IP) followed by Western blotting (WB) was performed by using the antibodies against Flag and Myc. The interaction indicates (a) PR209-FAM64A, (b) PR209-PLK3, (c) PR209-MPG, (d) PR209-HOXA1, (e) NT-HOXA1 and (f) CT-HOXA1. The lanes (1–3) represent: (1) input control of cell lysate, (2) IP with anti-Flag or anti-Myc antibody and (3) IP with normal mouse or rabbit IgG.

expressed as a Myc-tagged fusion protein. Coimmunoprecipitation and Western blot validated the interaction of PR209 with FAM64A (Figure 3a), PLK3 (Figure 3b), MPG (Figure 3c) and HOXA1 (Figure 3d). Furthermore, we found that not the N-terminal half but the C-terminal half of PR209 is bound to HOXA1 (Figure 3e,f), excluding non-specific coimmunoprecipitation of PR209 and the interactors in the transient expression system using HEK293 cells.

Next, PR209 was expressed as a DsRed-tagged fusion protein in HEK293 cells. It was located predominantly in the nucleus and the cytoplasm, and less abundantly on the plasma membrane (Figure 4, panels a, d, g, j, m). The EYFP-tagged fusion protein of FAM64A or HOXA1 was located predominantly in the nucleus, where it was colocalized with PR209 (Figure 4, panels b, c, h, i). The EYFP-tagged FAM64A protein was also located chiefly in the nucleus colocalized with DsRed-tagged PR209 in SK-N-SH cells similarly in HEK293, suggesting that the unique subcellular location of FAM64A and PR209 is not attributable to HEK293 cell-specific intracellular trafficking of the recombinant proteins (Figure 4, panels d–f). The GFP-tagged PLK3 fusion protein, expressed on the plasma membrane and in the cytoplasm, showed discernible colocalization with PR209 (Figure 4, panels k, l). The GFP-tagged MPG fusion protein was located chiefly in the nucleus, coexisting with PR209 (Figure 4, panels n, o). Thus, a substantial part of PRP209 and interactors are colocalized in specific subcellular compartments in HEK293 and SK-N-SH cells following transient expression.

Proteinase K sensitivity and detergent insolubility of PR209

To study the proteinase K-resistant property of PR209 coexpressed with the interactors in HEK293 cells, cellular protein extract was treated with proteinase K. In some experiments, the cells were exposed to MG-132 in the last 24 h before harvest. Coexpression of PR209 with HOXA1 or FAM64A did not generate proteinase K-resistant products regardless of treatment with MG-132 (Figure 5a, lanes 1–6; upper panel: HOXA1; lower panel: FAM64A). To determine the detergent-insoluble property of PR209, cellular protein extract was separated into 0.5% Nonidet P-40-soluble (S) and -insoluble (P) fractions. Unexpectedly, a great amount of the PR209 protein was recovered from the detergent-insoluble (P) fraction, even when PR209 alone without interactors was transiently expressed in HEK293 cells (Figure 5b, lanes 7, 8; upper panel: HOXA1; lower panel: FAM64A). The detergent-insoluble property of PR209 was not affected by coexpression of the interactors, such as HOXA1 or FAM64A, either in the presence or absence of MG-132 (Figure 5, lanes 9–12; upper panel: HOXA1; lower panel: FAM64A). Thus, coexpression of PR209 with the interactors did not produce proteinase K-resistant proteins, although PR209 showed an intrinsic detergent insolubility in HEK293 cells.

Molecular network analysis of PrPC interactors

Functional annotation based on DAVID showed that the great majority of PrPIPs identified by protein microarray analysis play a role in the recognition of nucleic acids, involved in regulation of diverse cellular function (Figure 6).

To identify the molecular network of PrPC and PrPIPs, we imported the list of Entrez gene IDs of 47 PrPIPs into KeyMolnet, the comprehensive biological information

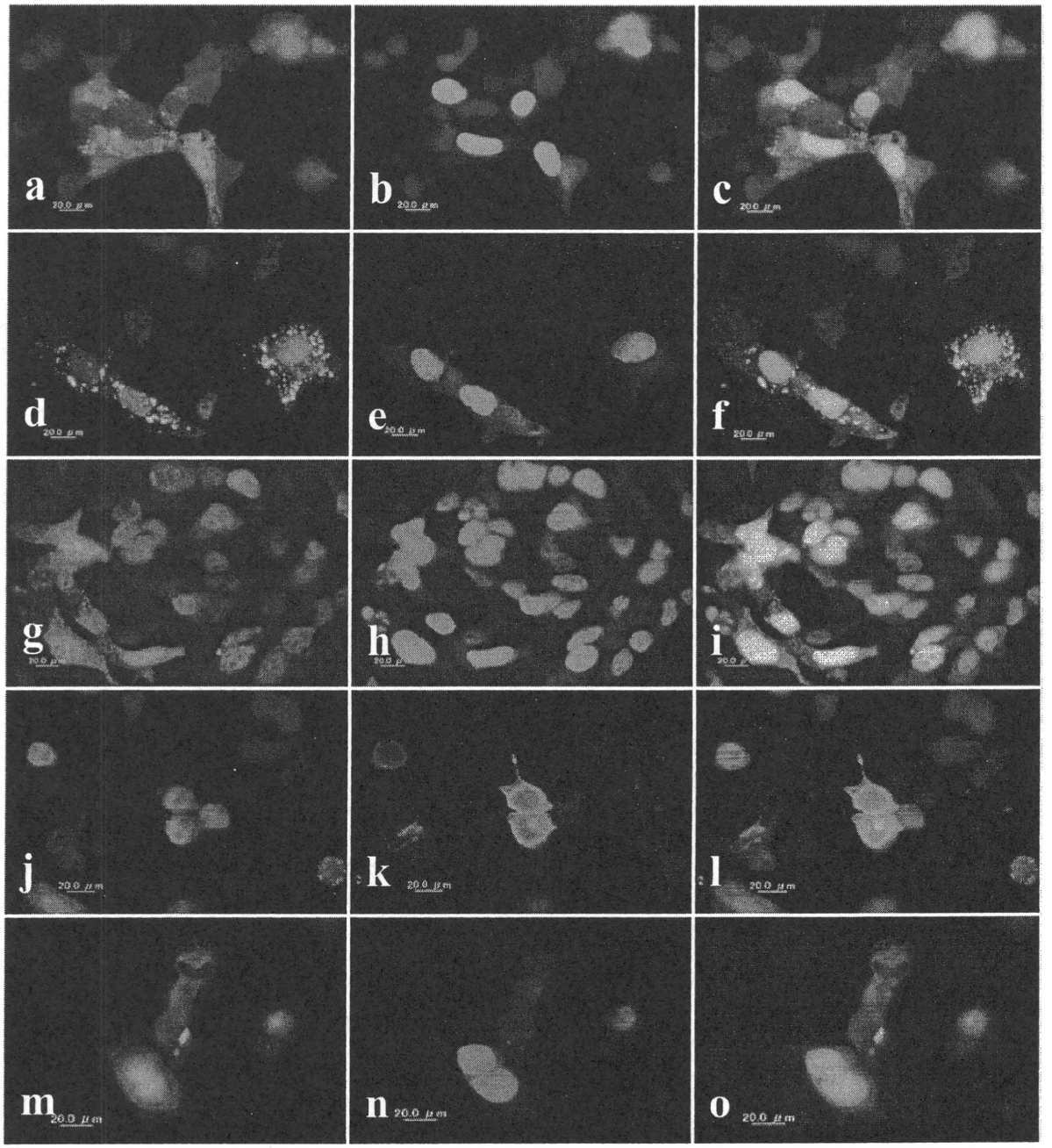


Figure 4. Cell imaging analysis. PR209 was expressed as a DsRed-tagged fusion protein, while FAM64A, HOXA1, PLK3 and MPG were expressed as an EYFP- or GFP-tagged fusion protein in HEK293 cells or in SK-N-SH cells. The panels (**a–o**) represent (**a–c** and **g–o**) HEK293 and (**d–f**) SK-N-SH of the following: (**a**) PR209, (**b**) FAM64A, (**c**) merge of **a** and **b**, (**d**) PR209, (**e**) FAM64A, (**f**) merge of **d** and **e**, (**g**) PR209, (**h**) HOXA1, (**i**) merge of **g** and **h**, (**j**) PR209, (**k**) PLK3, (**l**) merge of **j** and **k**, (**m**) PR209, (**n**) MPG and (**o**) merge of **m** and **n**.

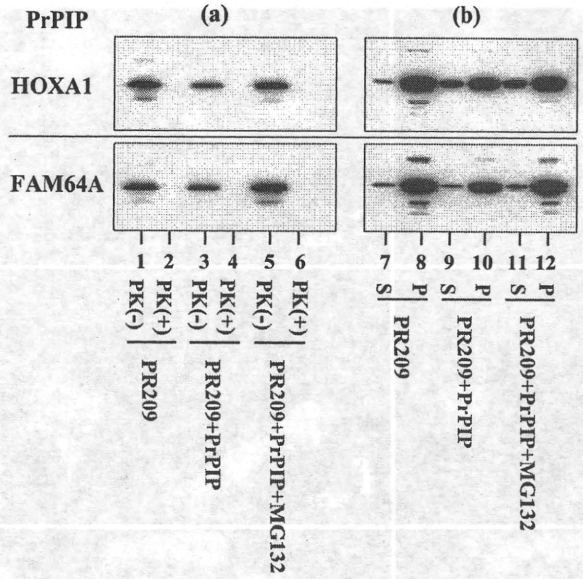


Figure 5. Biochemical property of PR209. Either Flag-tagged PR209 alone (lanes 1, 2, 7, 8) or the combination of PR209 and Myc-tagged PrPIP (lanes 3–6 and 9–12), such as HOXA1 (upper panels) or FAM64A (lower panels), were expressed in HEK293 cells. The cells were harvested at 48 h after transfection of the vectors. In some cultures, the cells were exposed to 10 μM MG-132 during the last 24 h before harvest (lanes 5, 6, 11, 12). (**a**) Proteinase K treatment. Total cellular protein extract was treated with (+; lanes 2, 4, 6) or without (–; lanes 1, 3, 5) 5 μg/ml proteinase K (PK) at 37°C for 30 min, and then processed for Western blot using 3F4 antibody. (**b**) Detergent treatment. Total cellular protein extract was separated into 0.5% Nonidet P-40-soluble (S: 50 μg of protein) and -insoluble (P: 7 μg of protein) fractions, and then processed for Western blot using 3F4 antibody.

platform of human molecules and molecular relations. It extracted 39 genes directly linked to 47 PrPIPs. Subsequently, the 'N-points to N-points' search starting from PrPC ending with 39 genes via the shortest route connecting them was performed. This generated a complex molecular network composed of 214 fundamental nodes and 579 molecular relations (Figure 7). Not surprisingly, KeyMolnet operating on the knowledgebase could not identify the direct interaction between PrPC and 47 PrPIPs, because their relationship has not been reported previously. Furthermore, KeyMolnet indicated the primary location of PrPC on the cell-surface membrane, but neither in the cytoplasm nor in the nucleus. When compared with the canonical pathways of KeyMolnet, statistical analysis indicated that the generated network has the most significant relationship with the AKT signalling pathway (the score 50.9). This was followed by the JNK signalling pathway in the second rank (the score 48.4), the MAPK signalling pathway in the third rank (the score 42.8) and the p38 signalling pathway in the fourth rank (the score 36.3). Thus, the molecular network of PrPC and interactors constitutes the key signal-transducing pathways pivotal for regulation of cell differentiation, proliferation, survival and apoptosis.

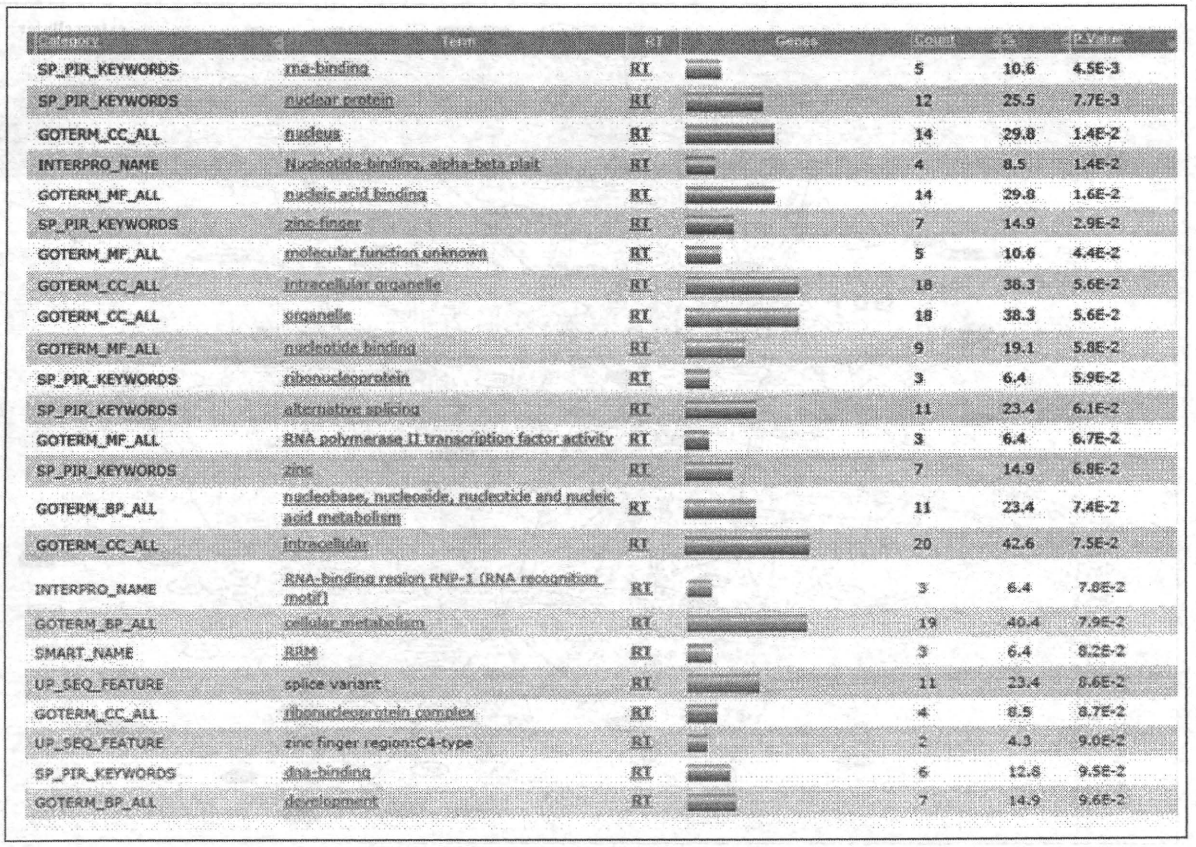
Discussion

We have performed screening of PrPIPs by using a human protein microarray containing 5000 proteins of various functional classes. By probing the array with PR209 spanning amino acid residues 23–231 of PrPC, we identified 47 novel PrPIPs. The functional annotation on the DAVID database suggested that the great majority of PrPIPs are categorized into the proteins involved in recognition of nucleic acids. The Allen Brain Atlas database search suggested that the great majority of 47 PrPIP orthologues are expressed in the adult rodent brain. Because high-throughput screening of high-density protein microarray enables us to identify a large number of putative binding partners at one time, it is often difficult to extract biological implications of their molecular relationship from such a large quantity of available data. To overcome this difficulty, we have made a breakthrough to identify the molecular network most closely associated with PrPC and the interactors by KeyMolnet, a bioinformatics tool for analysing molecular interaction on the curated knowledge database. The molecular network of PrPC and 47 PrPIPs on KeyMolnet showed an association most relevant to AKT, JNK and MAPK signalling pathways.

Advantages and limitations of protein microarray technology for identification of protein–protein interaction

Protein microarray serves as a powerful tool for the rapid and systematic identification of protein–protein and other biomolecule interactions. Protein microarray has a wide range of applications, including characterization of antibody specificity and autoantibody repertoire, and identification of novel biomarkers and molecular targets associated with disease type, stage and progression, leading to establishment of personalized medicine [47–49].

However, protein microarray technology is still under development in methodological aspects. In general,



Category	Term	RT	Genes	Count	%	PValue
SP_PIR_KEYWORDS	rna-binding	RT		5	10.6	4.5E-3
SP_PIR_KEYWORDS	nuclear protein	RT		12	25.5	7.7E-3
GOTERM_CC_ALL	nucleus	RT		14	29.8	1.4E-2
INTERPRO_NAME	Nucleotide-binding, alpha-beta plait	RT		4	8.5	1.4E-2
GOTERM_MF_ALL	nucleic acid binding	RT		14	29.8	1.6E-2
SP_PIR_KEYWORDS	zinc-finger	RT		7	14.9	2.9E-2
GOTERM_MF_ALL	molecular function unknown	RT		5	10.6	4.4E-2
GOTERM_CC_ALL	intracellular organelle	RT		18	38.3	5.6E-2
GOTERM_CC_ALL	organelle	RT		18	38.3	5.6E-2
GOTERM_MF_ALL	nucleotide binding	RT		9	19.1	5.8E-2
SP_PIR_KEYWORDS	ribonucleoprotein	RT		3	6.4	5.9E-2
SP_PIR_KEYWORDS	alternative splicing	RT		11	23.4	6.1E-2
GOTERM_MF_ALL	RNA polymerase II transcription factor activity	RT		3	6.4	6.7E-2
SP_PIR_KEYWORDS	zinc	RT		7	14.9	6.8E-2
GOTERM_BP_ALL	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	RT		11	23.4	7.4E-2
GOTERM_CC_ALL	intracellular	RT		20	42.6	7.5E-2
INTERPRO_NAME	RNA-binding region RNP-1 (RNA recognition motif)	RT		3	6.4	7.8E-2
GOTERM_BP_ALL	cellular metabolism	RT		19	40.4	7.9E-2
SMART_NAME	RRM	RT		3	6.4	8.2E-2
UP_SEQ_FEATURE	splice variant	RT		11	23.4	8.6E-2
GOTERM_CC_ALL	ribonucleoprotein complex	RT		4	8.5	8.7E-2
UP_SEQ_FEATURE	zinc finger region:C4-type	RT		2	4.3	9.0E-2
SP_PIR_KEYWORDS	dna-binding	RT		6	12.8	9.5E-2
GOTERM_BP_ALL	development	RT		7	14.9	9.6E-2

Figure 6. Functional annotation of PrPC interactors. Functional annotation of 47 PrPIPs identified by protein microarray analysis was performed by the program on DAVID bioinformatics database. When the list of Entrez gene IDs of 47 PrPIPs was imported, 34 genes were functionally categorized into 24 subgroups created by related databases, with enriched terms closely associated with the gene list examined. The genes excluded from the list ($n = 13$) are FAM64A, C7orf50, SCNM1, C18orf56, DKFZp761B107, TSLP, CWC15, KIAA1191, ARHGAP15, WDR5, WIBG, FAM27E3 and NOB1 (see Table 1 for the gene symbol). RT represents related term search. Genes and count indicate the genes involved in the term. The percentage is calculated from the formula following: gene involvement (%) = involved genes/total genes. *P*-value represents the *P*-value of gene enrichment analysis evaluated by the modified Fisher's exact test where it is the smaller, the genes are the more enriched in the term.

protein microarray has its own limitation associated with the expression and purification of a wide variety of target proteins. In the microarray we utilized, the target proteins were expressed in a baculovirus expression system, purified under native conditions, and spotted on to the slides to ensure the preservation of native structure, post-translational modifications such as glycosylation and phosphorylation [50], and proper functionality. In contrast, bacterially expressed proteins lack glycosylation and phosphorylation moieties, and are often misfolded during purification. As target proteins contain a GST fusion tag, the arrays are always processed for the post-spotting quality control by using an anti-GST antibody with a concentration gradient of GST spots as a standard. This procedure makes it possible to quantify the exact amount of proteins deposited in each spot, and thereby minimizes the inter-lot variability of the results. Furthermore, each subarray contains a series of built-in control spots.

Protein microarray also has another technical limitation attributable to the avidity of protein–protein interaction. The probing and rigorous washing procedure detects mostly the direct protein–protein interaction supported by the stable binding ability. It could not efficiently detect weak and transient protein–protein interactions, or indirect interactions that require accessory molecules or intervening cofactors. In addition, protein microarray screening does not consider the specific subcellular location where the protein–protein interaction actually takes

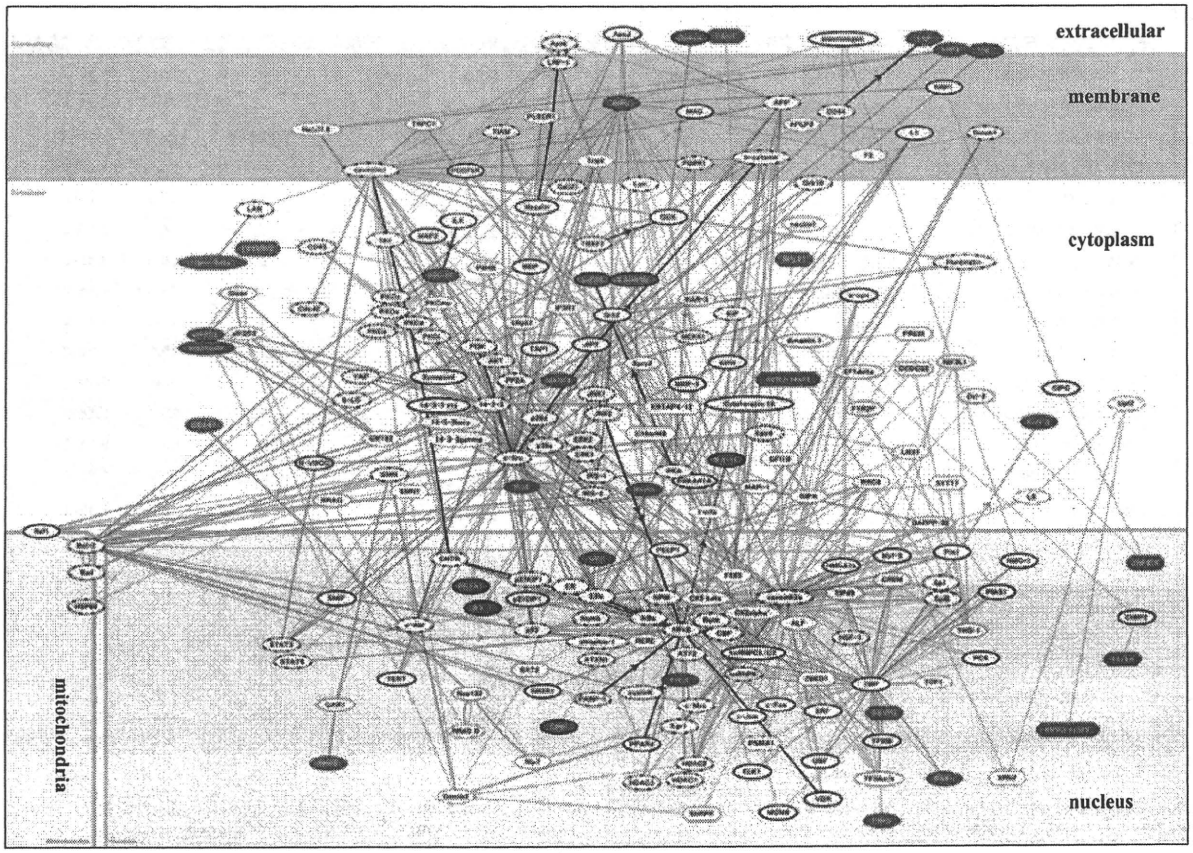


Figure 7. Molecular network of PrPC and the interactors. By importing the list of Entrez gene IDs of 47 PrPIPs, KeyMolnet extracted 39 genes directly linked to 47 PrPIPs. Subsequently, the 'N-points to N-points' search starting from PrPC ending with 39 genes generated a complex molecular network composed of 214 fundamental nodes and 579 molecular relations. They are arranged according to the predicted subcellular location. The red node indicates PrPC on the cell-surface membrane as the starting point, while blue nodes represent PrPIPs listed in Table 1. The connections of thick lines represent the core contents, while thin lines indicate the secondary contents of KeyMolnet. The molecular relation is indicated by dash line with arrow (transcriptional activation), solid line with arrow (direct activation) or solid line without arrow (direct interaction or complex formation).

place. Thus, it is possible that some promiscuous partners are detected, whereas some of the biologically important interactors *in vivo* are left beyond identification. Therefore, protein microarray data always require the validation by other independent methods such as coimmunoprecipitation, far Western blotting, the Y2H screening and so on. Post-translational modifications play a pivotal role in a range of protein–protein interactions. Immunolabelling of the array we utilized with anti-phosphotyrosine antibody showed that approximately 10–20% of the proteins on the array are phosphorylated (Invitrogen, unpubl. data). When the array was utilized for kinase substrate identification, most of known kinases immobilized on the array are enzymatically active with the capacity for autophosphorylation, suggesting that they are functionally active with preservation of proper conformation (data of Invitrogen).

Validation of interaction and colocalization of PrPC with four neuronal PrPC interactors by immunoprecipitation and cell imaging analysis

We selected four PrPIPs for further biochemical characterization, including FAM64A, HOXA1, PLK3 and MPG, because of their potential involvement in neural function. Furthermore, we identified the expression of all of these in differentiated human neurones NTera2N by RT-PCR. FAM64A is a 26-kDa protein with a DUF1466 domain in its N-terminal region. Currently, its biological function

remains unknown. However, the database search on Entrez UniGene, the organized view of the transcriptome, showed that the FAM64A transcript is expressed abundantly in brain tissues, glioma and primitive neuroectodermal tumours. HOXA1 acts as a transcription factor that regulates the proper arrangement of hindbrain segments during development [51]. Homozygous truncating mutations in the human HOXA1 gene disrupt brainstem, inner ear, cardiovascular and cognitive development in patients with the Bosley–Salih–Alorainy syndrome [52]. PLK3 is a member of the polo family serine/threonine kinases that regulate the onset of mitosis and M-phase progression in cell cycle. Long-term potentiation enhances PLK3 expression in hippocampal neurones, suggesting a role of PLK3 in synaptic plasticity [53]. MPG is a DNA repair enzyme that removes mutagenic alkylation adducts of purines from damaged DNA. Astrocytoma cells express a great amount of MPG protein, supporting a role of MPG in astrocytic tumorigenesis [54].

The interaction of PR209 with FAM64A, HOXA1, PLK3 and MPG was verified by coimmunoprecipitation and cell imaging in a transient expression system of HEK293 cells. Because the antibodies sufficient for immunoprecipitation with FAM64A, PLK3 and MPG are currently unavailable, we performed immunoprecipitation analysis by using the tag-specific antibodies. Although cultured human neurones, such as NTera2, appear to be preferable for expression of PrPC interactors, we utilized HEK293 cells because of much easier handling and constant expression of tagged recombinant proteins. It is worth noting that in preliminary experiments, we found that there exists a small but discernible level of interaction between endogeneous PrPC and HOXA1 in adult human brain tissue homogenates (data not shown).

PrPC is structurally separated into two distinct segments composed of the N-terminal flexibly disordered tail (amino acid residues 23–121) that includes the octapeptide repeat region, and the C-terminal globular domain (amino acid residues 121–230) that contains three α-helices and two short anti-parallel β-sheets [55]. The immunoprecipitation study showed that HOXA1 interacts exclusively with the C-terminal half of PR209.

PrPC interactors play a role in nuclear function

Although PrPC is a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein, we found that DsRed-tagged PR209 with the C-terminal GPI anchor site of amino acid residue 231 is located predominantly in the nucleus and the cytoplasm, and less abundantly on the plasma membrane. A recent study showed that PrPC after cleavage of both N-terminal and C-terminal signal peptides is located chiefly in the nucleus, where it interacts with chromatin in neural cell lines [56], supporting our findings that PrPC could interact with its partners in both the nucleus and the cytoplasm. PrPC has two cryptic nuclear localization signals in the N-terminal domain [57]. Nuclear localization of PrPSc-like protein is identified in prion-infected cells [58]. Furthermore, defined populations of neurones express PrPC in their cytoplasm [59]. In the present study, FAM64A, HOXA1 and MPG were located predominantly in the nucleus, where they coexisted with PR209. The involvement of PrPC and interactors in nuclear function is supported by functional annotation on the DAVID database that suggested a major role of PrPIPs in the recognition of nucleic acids. We could categorize the nucleic acid-binding PrPIPs into two distinct groups: (i) proteins involved in RNA splicing, silencing and metabolism: SCNM1, RBM22, DDX47, CWC15, PTRH1, EIF2C1, CIRBP, NOLA2, NOB1 and RNPC3, and (ii) proteins involved in DNA transcription and repair: HOXA1, BAZE2B, MPG, ZNF192, ZNF408, TBPL1, ABT1, ZNF740 and WDR5. Importantly, a previous study indicated that PrPC plays a key role in nucleic acid metabolism by its nucleic acid chaperoning activity [60]. Because protein microarray analysis utilized the recombinant PR209 highly purified from the culture supernatant as a probe, the possibility is unlikely that any contaminating cellular nucleic acids mediate the interaction between PrPC and PrPIPs.

Previous studies showed that PrPC spanning amino acid residues 23–230 designated CyPrP accumulates in the cytoplasm, where it is converted into the PrPSc-like proteinase K-resistant protein (PrP^{RES}) with potent neurotoxicity, when the proteasome activity is suppressed [61,62]. In contrast, we showed that coexpression of large amounts of PR209 with HOXA1 or FAM64A did not generate PrP^{RES} in HEK293 cells, even after exposure of the cells to MG-132. Our observations suggest that both HOXA1 and FAM64A do not act as the chaperone 'protein X' that promotes protein conformational conversion from PrPC to PrPSc at least in a short incubation time of 48 h. Because prion diseases develop after a long incubation period, our observations do not exclude the possibility that a long-term incubation of PrPC and the interactors with some additional cofactors could accelerate the conforma-

tional conversion from PrPC to PrPSc. In addition, we unexpectedly found that PR209 exhibits an intrinsic detergent insolubility in HEK293 cells following transient overexpression. A recent study showed that small amounts of detergent-insoluble prion protein aggregates are present in normal human brains [63].

The molecular network of PrPC and interactors involves key cell signalling pathways

KeyMolnet stores the comprehensive content database that focuses on human molecules and molecular interactions, carefully curated by experts from the literature and public databases [41]. This software makes it possible to effectively extract the most relevant molecular interaction from large quantities of gene expression data, and to establish a biologically relevant logical working model [42]. The present study for the first time by using KeyMolnet, a data-mining tool of bioinformatics, showed that the complex molecular network of PrPC and 47 PrPIPs has a significant relationship with AKT, JNK and MAPK signalling pathways. A previous study showed that PrPC activates diverse signalling pathways involving Fyn, PI3 kinase/Akt, cAMP-dependent protein kinase A and MAP kinase, all of which contribute to neurite outgrowth and neuronal survival in primary culture of mouse neurones [64]. PrPC-knockout mice show exacerbation of ischaemic brain injury, accompanied by reduced expression of Ser473-phosphorylated Akt and increased activities of ERK-1/–2, STAT-1 and caspase-3 in the brain [65,66]. A synthetic peptide PrP106–126 induces neuronal apoptosis in primary cultures of mouse neurones via the JNK-c-Jun pathway [67]. All of these observations suggest a crucial link between the biological function of PrPC and signalling pathways mediated by AKT, JNK and MAPK.

Previously, we showed that the genes located in the Ras/Rac signalling pathway, pivotal for cell proliferation, differentiation and survival, were aberrantly regulated in cultured fibroblasts of PrPC-deficient mice [68]. More recently, by analysing a DNA microarray containing 12 814 human genes, we identified 33 genes differentially expressed between a stable PrPC-expressing HEK293 cell line and the parent PrPC-non-expressing cells [37]. They included 18 genes involved in neuronal and glial functions, five related to production of the extracellular matrix, and two located in the complement cascade. These observations suggest that aberrant expression of PrPC, either overexpression or underexpression, affects a wide range of cell signalling pathways. Most recently, we showed that the zeta isoform of 14-3-3 protein, a scaffold protein on which diverse signal components converge, forms a molecular complex with PrPC and heat shock protein Hsp60 in the human CNS neurones under physiological conditions [23]. This raises the hypothesis that the multimolecular complex is disrupted in the pathological process of prion diseases, resulting in the release of 14-3-3 from degenerating neurones into the cerebrospinal fluid. Unfortunately, the protein microarray utilized in the present study does not include 14-3-3 zeta, Hsp60 or PrPC as targets.

In conclusion, protein microarray is a useful tool for systematic screening and comprehensive profiling of the human PrPC interactome. The great majority of PrPIPs are annotated as the proteins involved in the recognition of nucleic acids. Thus, individual PrPIPs possibly act as regulators of RNA splicing, silencing, and metabolism and modulators for DNA transcription and repair in neural and non-neural cells. Furthermore, the human PrPC–PrPIP network on the whole plays a pivotal role in signalling pathways essential for regulation of cell survival, differentiation, proliferation and apoptosis. These observations propose a logical hypothesis that the dysregultion of PrPC interactome might induce extensive neurodegeneration ongoing in prion diseases, and warrant further studies to clarify the implication of PrPC and the interactors in cellular signalling and nuclear function.

Acknowledgements

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Primers utilized for PCR in the present study.
Table S2. The complete list of the proteins immobilized on a human protein microarray utilized in the present study.

The supplementary material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2990.2008.00947.x

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Molecular network of the comprehensive multiple sclerosis brain-lesion proteome

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Background A recent proteomics study of multiple sclerosis (MS) lesion-specific proteome profiling clearly revealed a pivotal role of coagulation cascade proteins in chronic active demyelination. However, among thousands of proteins examined, nearly all of remaining proteins are yet to be characterized in terms of their implications in MS brain-lesion development.

Methods By the systems biology approach using four different pathway analysis tools of bioinformatics, we studied molecular networks and pathways of the proteome dataset of acute plaques, chronic active plaques (CAP), and chronic plaques (CP).

Results The database search on Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein analysis through evolutionary relationships (PANTHER) indicated the relevance of extracellular matrix (ECM)–mediated focal adhesion and integrin signaling to CAP and CP proteome. KeyMolnet disclosed a central role of the complex interaction among diverse cytokine signaling pathways in brain-lesion development at all disease stages, as well as a role of integrin signaling in CAP and CP. Ingenuity pathway analysis (IPA) identified the network constructed with a wide range of ECM components, such as collagen, type I α1, type I α2, type VI α2, type VI α3, fibronectin 1, fibulin 2, laminin α1, vitronectin, and heparan sulfate proteoglycan, as one of the networks highly relevant to CAP proteome.

Conclusions Although four distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin signaling in development of chronic lesions of MS. These *in silico* observations indicate that the selective blockade of the interaction between ECM and integrins in brain lesions *in situ* would be a target for therapeutic intervention in MS. *Multiple Sclerosis* 2009; **15:** 531–541. http://msj.sagepub.com

Key words: extracellular matrix; multiple sclerosis; pathway analysis; proteome; systems biology

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) presenting with relapsing-remitting and progressive clinical courses. An autoimmune process triggered by a complex interplay between genetic and environmental factors may mediate MS, although the causative agents have not yet been identified. Pathologically, MS shows remarkable heterogeneity in inflammatory demyelination, astrogliosis, and axonal degeneration [1]. Even though various drugs are lined up in clinical trials, currently, treatment options with limited efficacies, including interferon-β, glatiramer acetate, and mitoxantrone are available for ordinary clinical practice of MS [2].

The completion of the Human Genome Project in 2003 allows us to systematically characterize the comprehensive disease-associated profiles of the whole human genome [3]. The global analysis of transcriptome, proteome, protein interactome, and metabolome helps us identify disease-specific molecular signatures and biomarkers for diagnosis and prediction of prognosis, and would broaden the spectrum of molecular mechanism-based therapy for MS [4,5]. Actually, the comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes by DNA microarray identified a battery of genes aberrantly regulated in MS, whose role has not been previously predicted during its pathogenesis [6,7]. A recent proteomics study of MS lesion-specific proteome profiling showed

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that overproduction of tissue factor and protein C inhibitor plays a central role in molecular events ongoing in chronic active plaques (CAP) [8]. *In vivo* administration of coagulation cascade inhibitors really reduced the clinical severity in a mouse model of experimental autoimmune encephalomyelitis (EAE), supporting the view that the blockade of the coagulation cascade would be a potential approach for the treatment of MS [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins were left behind to be characterized in terms of their implications in MS brain-lesion development.

Since the global expression analysis of transcriptome and proteome usually produces high-throughput experimental data at a time, it is often difficult to find out the meaningful biological implications of the dataset. Recent advances in systems biology enable us to illustrate the cell-wide map of the complex molecular interactions by using the literature-based knowledgebase of molecular pathways [9,10]. In the scale-free molecular network, targeted disruption of limited numbers of critical components, on which the biologically important molecular connections concentrate, could disturb the whole cellular function by destabilizing the network [11]. From this point of view, the integration of comprehensive transcriptome and proteome data of disease-affected tissues with underlying molecular networks could provide the rational approach not only to characterize disease-relevant pathways but also to achieve the network-based choice of effective drug targets. By using four different pathway analysis tools of bioinformatics, this study was designed to characterize molecular networks and pathways of MS lesion-specific proteome data of Han, *et al.* [8]. Although the analysis by distinct platforms did not lead to fully identical results, they commonly suggested a role of extracellular matrix (ECM) and integrin signaling in chronic lesions of MS. These *in silico* observations indicate that ECM and integrins would be a target candidate for designing therapeutic intervention in MS.

Databases and methods

The dataset of the comprehensive MS brain-lesion proteome

In the original dataset of Han, *et al.* [8], fresh-frozen brain autopsy samples were collected from six MS patients of different clinical subtypes, acute, chronic, progressive, secondary progressive, or chronic progressive, with ages 27–54, and from two age-matched control subjects free of neurological diseases. The postmortem interval of each case ranged from 4 to 24 h. Multiple sclerosis lesions were classified into three distinct categories: acute plaques (AP), CAP (chronic active plaques), or chronic plaques (CP), based on histological evaluation of the disease activity, briefly as follows: AP showed characteristics of acute ongoing inflammation, edema, and active demyelination. CAP was characterized by chronic demyelination with active inflammation at the lesion edges, whereas CP represented chronic inactive demyelination accompanied by profound astrogliosis. Protein samples were prepared from small pieces of brain tissues isolated by laser-captured microdissection, and the tissue pieces were characterized separately by the standard histological examination. The proteins were separated on one-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. Then, the protein bands were dissected and digested in a gel with trypsin, and peptide fragments were processed for mass spectrometric analysis several times to obtain a saturation point. Among 2,574 proteins determined with high confidence, the application of a computational data exploration program named INTERSECT/INTERACT identified 158, 416, and 236 lesion-specific proteins that were detected exclusively in AP, CAP, and CP, respectively. In this study, we tentatively called them as the comprehensive MS brain-lesion proteome dataset.

Conversion of protein IDs into Entrez Gene IDs and KEGG IDs

We converted the protein IDs listed in the dataset described above into the corresponding the National Center for Biotechnology Information (NCBI) Entrez Gene IDs, Gene Symbols, and Kyoto Encyclopedia of Genes and Genomes (KEGG) IDs by searching them on the UniProt knowledgebase (http://www.expasy.org/sprot).

Molecular network analysis

To identify biologically relevant molecular pathways from large-scale proteome data, we have undertaken the systems biology approach. We analyzed them by using four distinct pathway analysis tools endowed with a comprehensive knowledgebase which are as follows: KEGG (http://www.kegg.jp), the protein analysis through evolutionary relationships (PANTHER) classification system (http://www.pantherdb.org), Ingenuity pathways analysis (IPA) (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com), and KeyMolnet (Institute of Medicinal Molecular Design, Tokyo, Japan; http://www.immd.co.jp).

By importing the list of KEGG IDs, we studied molecular pathways on KEGG, a public database that systematically integrates genomic and chemical information to create the whole biological system *in silico*. KEGG contains manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases [12]. Currently, KEGG contains 90,931 pathways generated from 371 reference pathways. PANTHER, a public database generated by computational algorithms that relate the evolution of protein sequence to the evolution of protein functions and biological roles, provides a structured representation of protein function in the context of biological reaction networks [13]. Currently, PANTHER includes the information on 165 regulatory and metabolic pathways, manually curated by expert biologists. PANTHER visualizes pathway maps with the format compatible with the Systems Biology Markup Language (SBML) standard. By uploading the list of Entrez Gene IDs, PANTHER identifies the genes in terms of over- or under-representation in canonical pathways, followed by statistical evaluation by multiple comparison with a Bonferroni correction.

IPA is a commercial tool built upon a knowledgebase that contains approximately 1,600,000 biological and chemical interactions and functional annotations with scientific evidence. They are collected from more than 300 selected articles, textbooks, and other data sources, manually curated by expert biologists. By uploading the list of Entrez Gene IDs, the network-generation algorithm identifies focused genes integrated in a global molecular network [14]. IPA calculates the score *P*-value, the statistical significance of association between the genes and the network by the Fisher's exact test.

KeyMolnet is a commercial database, composed of knowledge-based contents on relationships among human genes, molecules, diseases, pathways, and drugs, curated by expert biologists. They are categorized into the core contents that are collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of PubMed database and Human Reference Protein database. By importing the list of Entrez gene ID, KeyMolnet automatically provides corresponding molecules as a node on networks [15]. The "N-points to N-points" network-search algorithm identifies the molecular network constructed by the shortest route connecting the start point molecules and the end point molecules. The generated network was compared side by side with 346 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations composed of approximately 90,000 sets, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p))$$

$$\text{Score}(p) = \sum_{x=0}^{\text{Min}(C,V)} f(x)$$

$$f(x) = {}_C C_x \cdot {}_{T-C} C_{V-x} / {}_T C_V$$

Results

KEGG and PANTHER searches elucidated a role of ECM-mediated cell adhesion in chronic lesions of MS

First of all, we converted all protein IDs listed in the original database [8] into the corresponding NCBI Entrez Gene IDs, Gene Symbols, and KEGG IDs by searching them on the UniProt knowledgebase. After the removal of unaccepted and redundant IDs, we finally identified 155, 407, and 232 Entrez Gene IDs and KEGG IDs from the AP, CAP, and CP-specific proteome data, respectively. They are listed in Supplementary Tables 1–3*.

When the KEGG IDs of the proteome were uploaded onto the 'Search Objects in Pathway' tool of the KEGG database, the vast majority of AP, CAP, or CP-specific proteins was not mapped on any KEGG human reference pathways (Table 1). However, a battery of CAP-specific proteins were categorized as those located in the pathways linked to focal adhesion (KEGG pathway ID: hsa04510), cell communication (hsa01430), ECM-receptor interaction (hsa04512), purine metabolism (hsa00230), and other biological pathways (not shown). Likewise, a panel of CP-specific proteins was found to be involved in the pathways linked to focal adhesion, regulation of actin cytoskeleton (hsa04810), oxidative phosphorylation (hsa00190), and cell communication (Table 1). These results are derived chiefly from enhanced production and deposition of ECM and receptor components, including collagen, fibronectin, vitronection, integrin, and laminin in CAP and CP lesions. In contrast, relatively small numbers of AP-specific proteins were mapped on the

*Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

Table 1 The molecular pathway relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KEGG search

Stage	Rank	Functional category (KEGG Pathway ID)	Genes classified
AP	1	Unclassified	123 genes
	2	Oxidative phosphorylation (hsa00190)	NDUFS7, NDUFB9, ATP4A, ATP6V0C
	3	Regulation of actin cytoskeleton (hsa04810)	FGD1, ITGB4, SSH1, ACTA1
CAP	1	Unclassified	281 genes
	2	Focal adhesion (hsa04510)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, MYLK, SHC3, PPP1CA, PARVA, PRKCB1, MYL7, RAC3, SPP1, SRC, THBS1, VTN
	3	Cell communication (hsa01430)	NES, COL1A, COL1A2, COL5A2, COL6A2, COL6A3, KRT78, FN1, GJA1, LAMA1, KRT3, SPP1, THBS1, VTN
	4	ECM-receptor interaction (hsa04512)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, HSPG2, SPP1, THBS1, VTN
	5	Purine metabolism (hsa00230)	ADCY5, TYMP, NT5E, PDE2A, PDE3B, PDE4A, PDE4B, PRPS2, GMPS, ENTPD1
CP	1	Unclassified	166 genes
	2	Focal adhesion (hsa04510)	COL4A2, COL6A1, CRK, FYN, ITGA6, LAMB2, LAMC1, PIK3CA, ZYX
	3	Regulation of actin cytoskeleton (hsa04810)	WASF2, BAIAP2, CRK, ITGA6, PIK3CA, TIAM1, MYH14, ARHGEF7
	4	Oxidative phosphorylation (hsa00190)	NDUFB6, NDUFB8, NDUFS5, ATP5I, ATP6V1F
	5	Cell communication (hsa01430)	COL4A2, COL6A1, ITGA6, LAMB2, LAMC1

The list of KEGG IDs of MS brain-lesion proteome was uploaded onto the 'Search Objects in Pathway' tool of the KEGG database. Top 2 for AP and top 4 for CAP and CP of human reference pathways relevant to the proteome data are shown with KEGG pathway IDs and the list of genes classified.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

pathways, such as oxidative phosphorylation and regulation of actin cytoskeleton (Table 1). Thus, the KEGG search suggested that the biological process of ECM and integrin-mediated cell adhesion and communication plays a role in chronic lesions of MS.

When the Entrez Gene IDs of the proteome were imported into the 'Gene Expression Data Analysis' tool of the PANTHER database, the vast majority of AP, CAP, or CP-specific proteins were not mapped on any PANTHER canonical pathways in comparison with a reference set of NCBI human genes (Table 2). However, PANTHER identified a statistically significant relationship between a set of CAP proteins and signaling pathways of chemokines and cytokines, integrin (Figure 1), muscarinic and nicotinic acetylcholine receptors (Table 2). PANTHER suggested an involvement of integrin signaling in CP, but identified no pathways relevant to AP (Table 2). Thus, the PANTHER search indicated that integrin signaling plays a role in both CAP and CP, whereas inflammation mediated by chemokine and cytokine signaling plays a predominant role in CAP.

Table 2 The molecular pathway relevant to MS brain-lesion proteome suggested by PANTHER search

Stage	Rank	Functional category	Number of genes classified	Human reference genes	*P*-value
AP	1	Unclassified	120	22436	6.89E-02 (NS)
CAP	1	Unclassified	321	22436	1,73E-04
	2	Inflammation mediated by chemokine and cytokine signaling pathway	17	315	2,63E-03
	3	Integrin signaling pathway	14	227	3,55E-03
	4	Muscarinic acetylcholine receptor 1 and 3 signaling pathway	7	62	1,17E-02
	5	Nicotinic acetylcholine receptor signaling pathway	8	91	2,03E-02
CP	1	Unclassified	182	22436	9,75E-03
	2	Integrin signaling pathway	9	227	4,33E-02

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing with a reference set of NCBI human genes. The canonical pathways relevant to the proteome data are shown with the number of genes classified and *P*-value evaluated by multiple comparison with a Bonferroni correction.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; and NS, not significant.

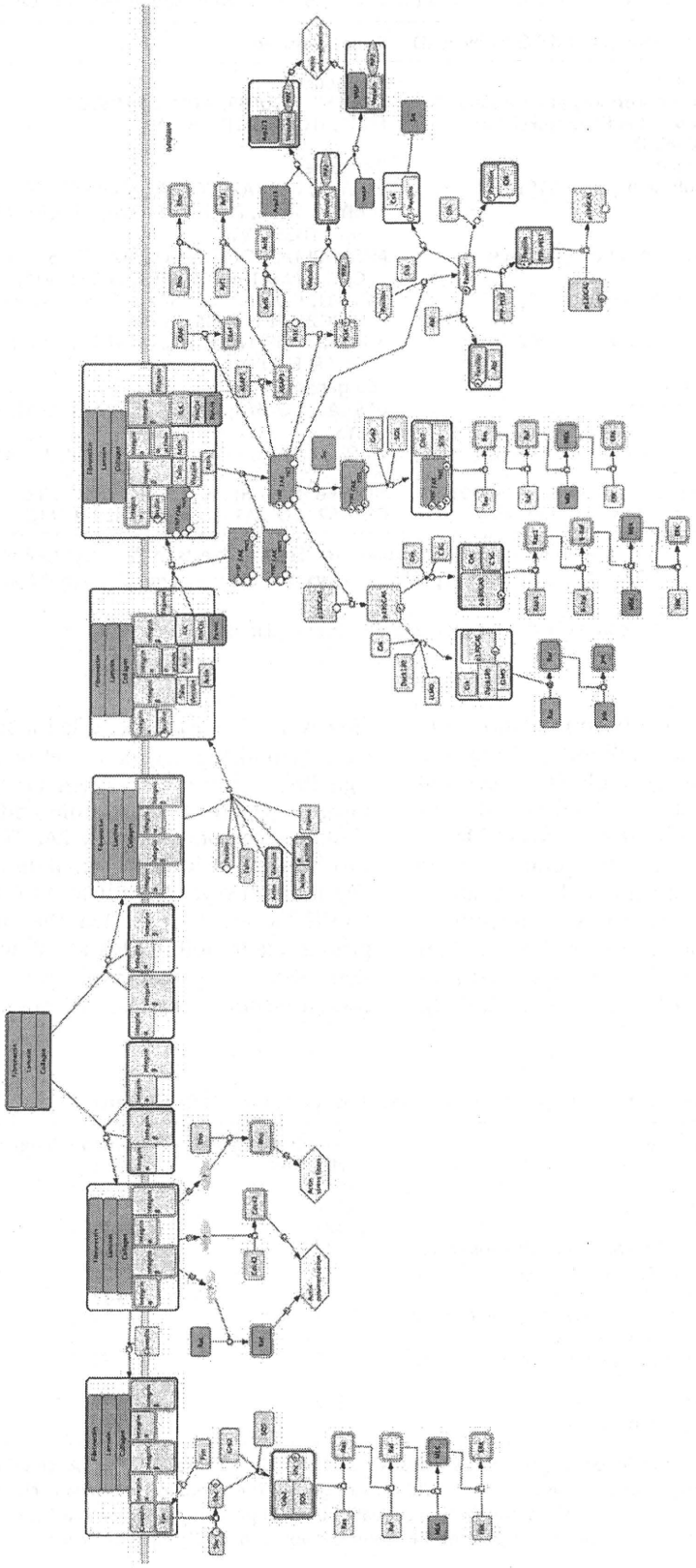

Figure 1 Integrin signaling pathway relevant to CAP proteome suggested by PANTHER. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing them with a reference set of NCBI human genes. Integrin signaling pathway was identified as one of canonical pathways statistically relevant to the CAP proteome (Table 2). The pathway is illustrated as the map compatible with the Systems Biology Markup Language (SBML) standard. The molecules colored in pink represent those included in the gene list (Supplementary Table 2). They are composed of fibronectin (Gene symbol: FN1), laminin (LAMA1), collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), Rac (RAC3), MEK (MAP2K4), FAK (PTK2B), parvin (PARVA), Src (SRC), Jnk (MAP2K4), Arp2/3 (ARPC1A), and VASP (ENAH).

KeyMolnet and IPA searches disclosed a role of the complex interaction of diverse intracellular signaling pathways in brain lesion development of MS

Next, we investigated molecular networks of MS brain proteome by utilizing two different commercial platforms. When the Entrez Gene IDs of the proteome were uploaded onto the "N-points to N-points" search tool of KeyMolnet, it extracted highly complex large-scale molecular networks of the AP, CAP, and CAP-specific proteome (Figure 2). The network of the AP, CAP, or CP proteome is composed of 777, 1,120, or 952 fundamental nodes with 1,892, 2,772, or 2,279 molecular relations, respectively. The statistical evaluation indicated that the top five most relevant molecular networks include IL-4, IL-6, IL-2, and catenin signaling pathways and transcriptional regulation by STAT (signal transducer and activator of transcription) for the AP proteome, PI3K, IL-4, type I IFN, and IL-6 signaling pathways and transcriptional regulation by STAT for the CAP proteome, and IL-4, hepatocyte growth factor (HGF), TCR (T cell receptor), integrin and IL-6 signaling pathways for the CP proteome (Table 3). It is worthy to note that the integrin signaling pathway was ranked as the sixth relevant pathway to the CAP proteome with *P*-value of the score = 2.13E-012. Considerable overlap existed in the results of PANTHER (Table 2) and KeyMolnet (Table 3). The KeyMolnet search disclosed a central role of the complex interaction of diverse cytokine signaling pathways in brain lesion development at all disease stages of MS, and the role of the integrin signaling pathway in both CAP and CP.

When the Entrez Gene IDs of the proteome were imported into the 'Core Analysis' tool of IPA, it highlighted several units of small-scale molecular networks relevant to the proteome data (Table 4). The network most relevant to the AP proteome was linked to the functional category of cellular assembly and organization, cancer, and cellular movement with the score *P*-value = 1.00E-49, where both ERK (extracellular signal-regulated kinase) and Akt (V-akt murine thymoma viral oncogene homolog) act as a hub of the network with highly connected molecular relations (Figure 3A). The network most relevant to the CAP proteome included two categories with the score *P*-value = 1.00E-47. One is the network of dermatological diseases and conditions, connective tissue disorders, and inflammatory disease. This network is constructed with various ECM components, including collagen, type I α1, type I, α2, type VI α2, type VI α3, fibronectin 1, fibulin 2, laminin α1, vitronectin, and heparan sulfate proteoglycan, where ERK acts as a hub (Figure 3B). The other is the network of lipid metabolism, molecular transport, and small molecule biochemistry, where Akt acts as a hub (Figure 3C). The network most relevant to the CP proteome was linked to cell cycle, cell morphology, and cell-to-cell signaling and interaction with the score *P*-value = 1.00E-50, where NF-κB (nuclear factor-kappa B) serves as a hub (Figure 3D). Overall, the biological processes involved in cellular assembly, organization, growth, proliferation, movement, and development are key functional categories shared by AP and CP molecular networks (Table 4). IPA also identified in the canonical pathways relevant to the proteome data. Both calcium signaling and oxidative phosphorylation were categorized as those relevant to AP and CAP proteome, whereas the actin cytoskeleton signaling pathway was considered as the important pathway in both CAP and CP (Table 5). Considerable overlap existed in the results of KEGG (Table 1) and IPA (Table 5).

Discussion

A recent proteomics study of MS lesion-specific proteome profiling clearly showed a pivotal role of coagulation cascade proteins in chronic active demyelination [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins are left behind to be characterized in terms of their implications in MS brain-lesion development. The present study characterized molecular networks and pathways of the proteome data by using four different pathway analysis tools of bioinformatics. Although distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin-mediated signaling as the pathway relevant to chronic lesion of MS. Therefore, these *in silico* observations warrant experimental validation.

In the CNS, ECM proteins provide a microenvironment for neurons and glial cells to maintain the ionic and nutritional homeostasis. They are localized chiefly to the vascular and the astroglial basement membranes and meninges but scarcely found in the brain parenchyma under physiological conditions. ECM proteins interact with integrins, the cell-surface ligands that support a physical link between ECM and cytoskeletal components [16]. Integrins consist of 24 pairs composed of noncovalently linked heterodimeric αβ subunits. Although the interaction between integrins and ECM proteins is partially redundant, β1 integrins are the principal ligand for collagen, fibronectin, and laminin, whereas αv integrins are the primary ligand for vitronectin. Integrins regulate the cytoskeletal rearrangement required for cell growth, movement, proliferation, and differentiation by transducing bidirectional signals in an 'inside-out' and 'outside-in' fashion [16]. Integrins, expressed on

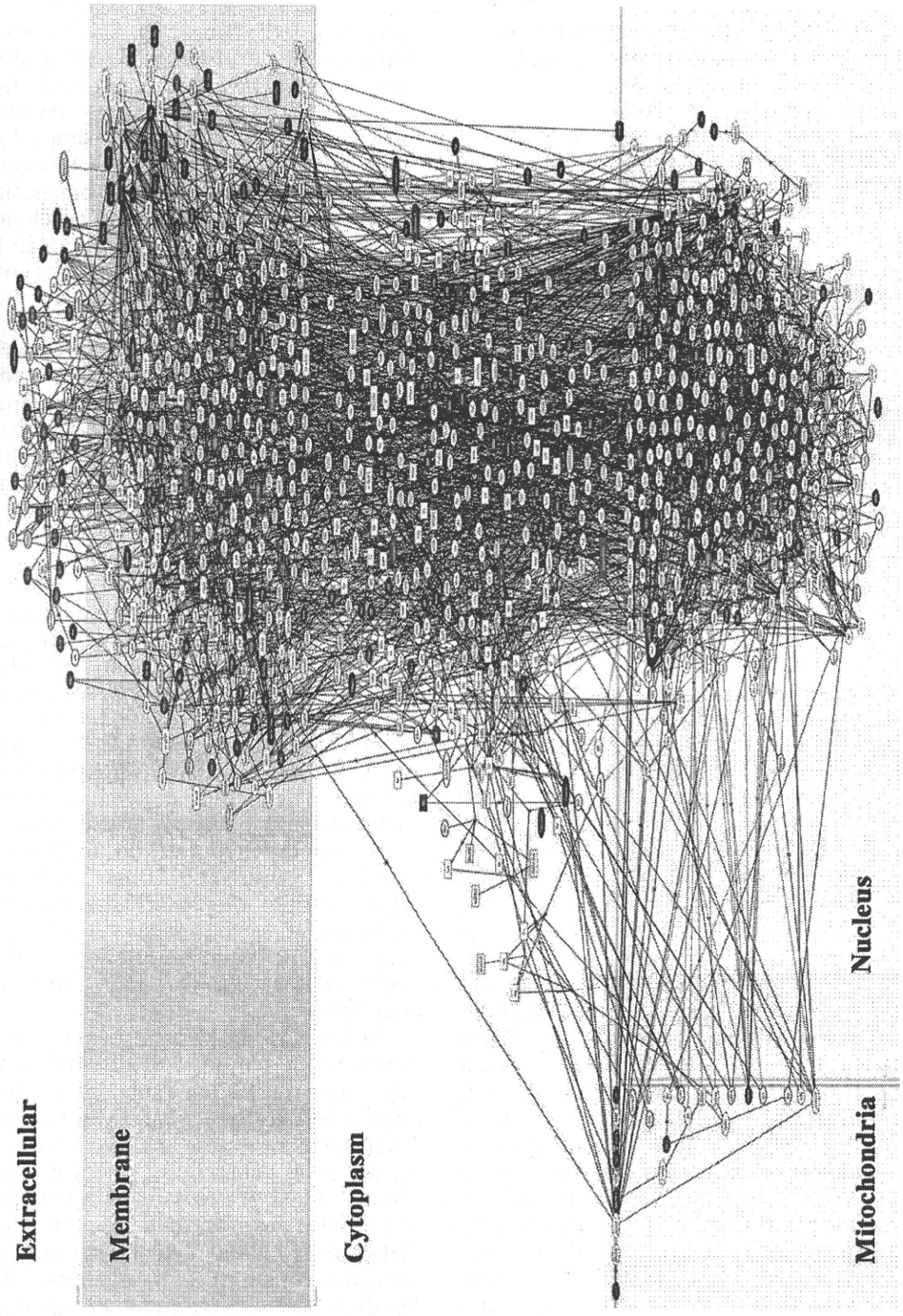

Figure 2 The molecular network of the CAP proteome suggested by KeyMolnet. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. This generated a complex network composed of 1,120 fundamental nodes with 2,772 molecular relations, constructed by the shortest route connecting the start point of 75 MS-linked molecules of the KeyMolnet library (Supplementary Table 4)* and the end point of the CAP-specific proteome. The network is illustrated with respect to subcellular location of molecules. Red nodes represent start point molecules, whereas blue nodes represent end point molecules. Purple nodes express characteristics of both start and end point molecules. White nodes exhibit additional molecules extracted automatically from KeyMolnet core contents to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). *Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

Table 3 The molecular network relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KeyMolnet search

Stage	Rank	Functional category	Score	*P*-value
AP	1	IL-4 signaling pathway	42,324	1,794E-13
	2	IL-6 signaling pathway	40,966	4,656E-13
	3	IL-2 signaling pathway	36,684	9,059E-12
	4	Transcriptional regulation by STAT	32,789	1,347E-10
	5	Catenin signaling pathway	32,725	1,408E-10
CAP	1	PI3K signaling pathway	56,937	7,25E-18
	2	IL-4 signaling pathway	46,914	7,541E-15
	3	Transcriptional regulation by STAT	43,694	7,025E-14
	4	IFNα/βsignaling pathway	41,557	3,09E-13
	5	IL-6 signaling pathway	41,274	3,762E-13
CP	1	IL-4 signaling pathway	53,096	1,039E-16
	2	HGF signaling pathway	45,735	1,708E-14
	3	TCRα/βsignaling pathway	43,621	7,39E-14
	4	Integrin signaling pathway	38,501	2,572E-12
	5	IL-6 signaling pathway	38,115	3,359E-12

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. The molecular network is constructed by the shortest route connecting the start point of 75 MS-related molecules of the KeyMolnet library (Supplementary Table 4) and the end point of MS lesion–specific proteome. Top 5 networks relevant to the proteome data are shown with the score and *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; PI3K, phosphoinositide-3-kinase; and HGF, hepatocyte growth factor.

immune cells, act as an adhesion receptor for cell trafficking and serve as a scaffold for immunological synapses. By the KEGG search, we identified focal adhesion, cell communication, and ECM-receptor interaction as molecular pathways most relevant to the CAP proteome. They involve a wide range of ECM components, including collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), fibronectin (FN1), laminin (LAMA1), vitronectin (VTN), heparan sulfate proteoglycan (HSPG2), thrombospondin (THBS1), parvin (PARVA), and osteopontin (SPP1). Furthermore, we found focal adhesion, regulation of actin cytoskeleton, and cell communication as the pathways involved in CP. They include collagen (COL4A2, COL6A1), laminin (LAMB2, LAMC1), and integrin (ITGA6). The relevance of

Table 4 The molecular network relevant to multiple sclerosis (MS) brain-lesion proteome suggested by IPA search

Stage	Rank	Functional category	The number of genes classified	*P*-value
AP	1	Cellular assembly and organization; cancer; cellular movement	24	1,00E-49
	2	Small molecule biochemistry; molecular transport; cellular assembly and organization	15	1,00E-26
	3	Cellular assembly and organization; cellular function and maintenance; skeletal and muscular system	14	1,00E-24
	4	Cellular development; cellular growth and proliferation; hematological system development and function	13	1,00E-22
	5	Cellular compromise; immune and lymphatic system development and function; hair and skin development and function	12	1,00E-19
CAP	1	Dermatological diseases and conditions; connective tissue disorders; inflammatory disease	29	1,00E-47
	2	Lipid metabolism; molecular transport; small molecule biochemistry	29	1,00E-47
	3	Cardiovascular disease; nephrosis; renal and urological disease	25	1,00E-38
	4	Endocrine system disorders; metabolic disease; renal and urological disease	25	1,00E-38
	5	Skeletal and muscular system development and function; tissue morphology; cardiovascular system development and function	22	1,00E-31
CP	1	Cell cycle; cell morphology; cell–cell signaling and interaction	27	1,00E-50
	2	Tissue morphology; cardiovascular disease; cellular development	24	1,00E-43
	3	Cellular assembly and organization; cell morphology; cellular movement	22	1,00E-38
	4	Cellular assembly and organization; cellular development; cellular growth and proliferation	18	1,00E-29
	5	Cell–cell signaling and interaction, Hematological system development and function; Immune and lymphatic system development and function	15	1,00E-22

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Core Analysis' tool of IPA. Top five molecular networks relevant to the proteome data are shown with the number of genes classified and the score *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

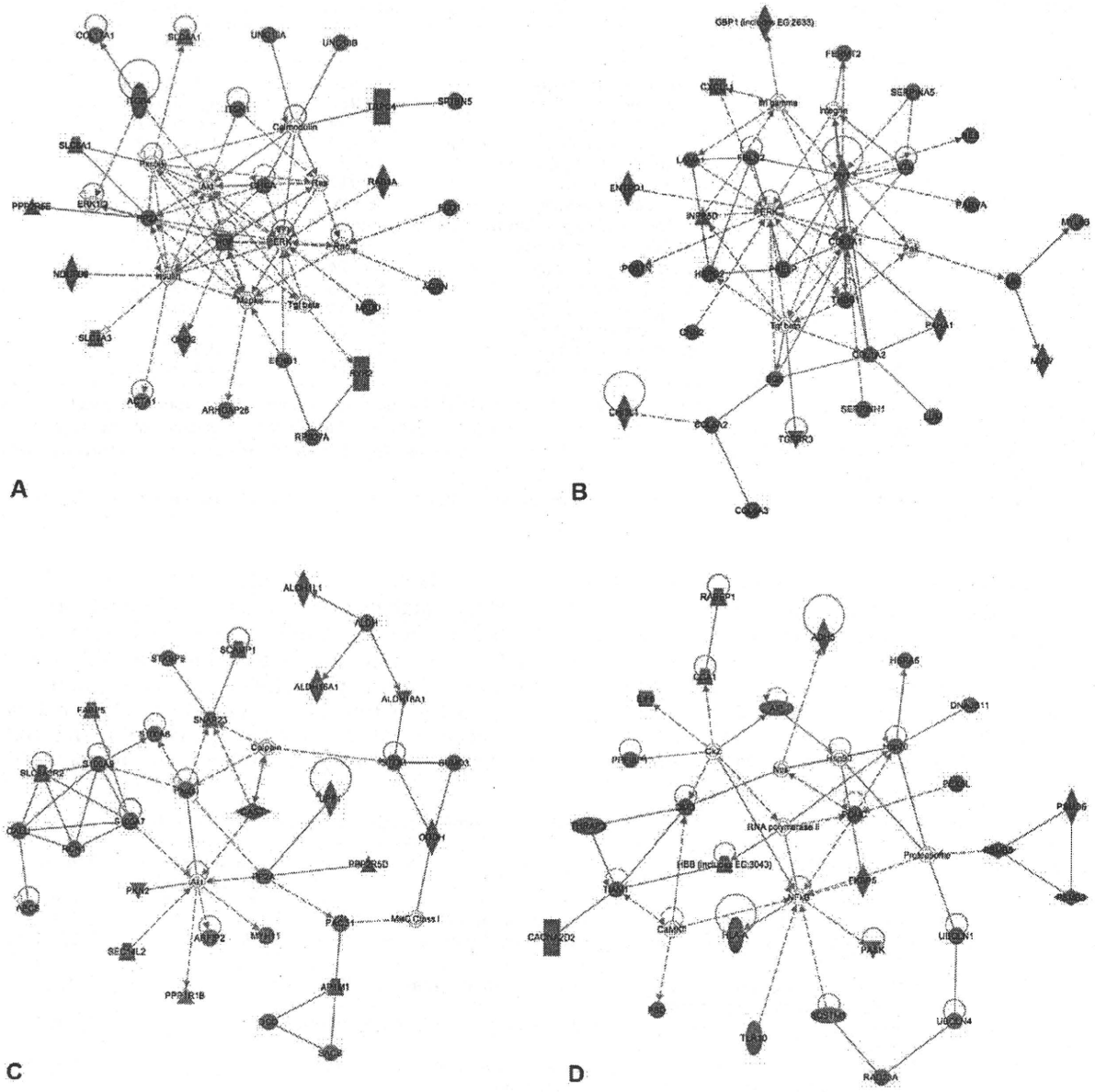

Figure 3 The molecular network of the AP, CAP, and CP proteome suggested by IPA. The list of Entrez Gene IDs of the MS lesion–specific proteome was uploaded onto the 'Core Analysis' tool of Ingenuity pathway analysis (IPA). Molecular networks most relevant to the AP (A), CAP (B and C), or CP (D) proteome are shown. Red nodes represent the molecules included in the gene list (Supplementary Tables 1–3). The molecular network (A) is constructed by 35 nodes, including ACTA1, AGRN, Akt, ARHGAP26, Calmodulin, CHD2, CHGA, COL17A1, EFNB1, ERK, ERK1/2, FGD1, HGF, insulin, ITGB4, ITSN1, MADD, Mapk, NDUFB9, Pkc(s), PP2A, PPP2R5E, RAB1A, Rac, Ras, RPS27A, RYR2, SLC2A3, SLC6A1, SLC8A1, SPTBN5, TGF-β, TRPC4, UNC13A, and UNC13B. The network (B) is constructed by 35 nodes, including BGN, CHI3L1, CNN2, COL1A1, COL1A2, COL6A2, COL6A3, CXCL11, ENTPD1, ERK, FBLN2, FERMT2, FN1, GBP1, HSPG2, IFN-γ, INPP5D, Integrin, LAMA1, LUM, Mlc, MYL7, MYL6B, NES, P4HA1, Pak, PARVA, POSTN, PRELP, SERPINA5, SERPINH1, TGF-β, TGFBR3, THBS1, and VTN. The network (C) is constructed by 35 nodes, including Akt, ALDH, ALDH16A1, ALDH18A1, ALDH1L1, AP1M1, APCS, ARFIP2, Calpain, CALU, CAST, DCD, FABP5, MHC Class I, MYH11, OGDH, PACS1, Pkc(s), PKN2, PP2A, PPP1R1B, PPP2R5D, RCN1, S100A7, S100A8, S100A9, SACS, SCAMP1, SEC14L2, SLC9A3R2, SNAP23, STOM, STXBP5, SUMO3, and UPF1. The network (D) is constructed by 35 nodes, including ADH5, AIP, CACNA2D2, CaMKII, Ck2, DMD, DNAJB11, EIF5, FKBP5, GGA1, HBB, HLA-A, Hsp70, Hsp90, HSPA6, NFkB, Nos, PASK, PEX5L, POMC, PPFIBP1, Proteasome, PSD, PSMB3, PSMB5, PSMD6, RABEP1, RAD23A, RNA polymerase II, SQSTM1, THRAP3, TIAM1, TLR10, UBQLN1, and UBQLN4. The molecular relation is indicated by solid line (direct interaction), dash line (indirect interaction), with filled arrow (acts on), stop (inhibits), stop and filled arrow (inhibits and acts on), and open arrow (translocates to).

Table 5 The molecular pathway relevant to MS brain-lesion proteome suggested by Ingenuity pathway analysis (IPA) search

Stage	Rank	Functional category	The number of genes classified	*P*-value
AP	1	Calcium signaling	7	2,53E-03
	2	Oxidative phosphorylation	4	2,69E-02
CAP	1	Calcium signaling	14	5,14E-04
	2	Hepatic fibrosis and hepatic stellate cell activation	11	1,53E-03
	3	Purine metabolism	16	3,05E-03
	4	Actin cytoskeleton signaling	13	5,77E-03
	5	Oxidative phosphorylation	9	1,12E-02
CP	1	Biosynthesis of steroids	4	7,37E-04
	2	Actin cytoskeleton signaling	8	8,00E-03
	3	Ubiquinone biosynthesis	4	9,54E-03
	4	Axonal guidance signaling	11	1,37E-02
	5	Integrin signaling	7	2,19E-02

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Core Analysis' tool of IPA. The canonical pathways relevant to the proteome data are shown with the number of genes classified and *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

the ECM and integrin signaling pathway to CAP and CP was further verified by molecular network analysis using PANTHER, KeyMolnet, and IPA followed by statistical evaluation. These *in silico* observations agree well with *in-vivo* studies, showing remarkable upregulation of diverse ECM constituents in MS brain lesions, where cytokine/chemokine-activated microglia, astrocytes, and infiltrating macrophages release a large amount of proteolytic enzymes bound to ECM molecules, which mediate myelin breakdown [17,18]. Glial scars in chronic lesions of MS include certain ECM proteins that contribute to the failure of regeneration of damaged axons and remyelination of preserved axons [17,18].

In active demyelinating lesions of MS, the expression of vitronectin is greatly enhanced in blood vessel walls, as well as in demyelinated axons and hypertrophic astrocytes at the edge of demyelination [19]. The levels of CD51, a vitronectin receptor, are elevated in the serum of relapsing-remitting MS patients [20]. Vitronectin promotes migration of reactive astrocytes expressing αvβ8 integrin [21]. In active demyelinating lesions of MS, fibronectin is accumulated in the brain parenchyma and is deposited abundantly in blood vessel walls and perivascular infiltrates [22]. Fibronectin facilitates migration of immune cells, promotes proliferation of astrocytes, and inhibits differentiation of oligodendrocyte progenitors [23]. In MS lesions, both vitronectin and fibronectin are derived mainly form plasma protein components passing across the disrupted blood–brain barrier and partly from the local synthesis by endothelial cells, macrophages, astrocytes, and infiltrating immune cells. Vitronectin and fibronectin activate microglia and upregulate MMP-9 production [24]. Thrombospondin produced by reactive astrocytes facilitates macrophage-mediated phagocytosis of apoptotic cells and possible uptake of degraded myelin via the ECM receptors CD36 and αvβ3 integrin [25]. Large-scale sequencing of MS plaque cDNA libraries showed that osteopontin (SPP1), a proinflammatory component of ECM, is one of the most abundant transcripts [26]. The clinical severity of EAE is attenuated in SPP1-deficient mice [26]. The expression of osteopontin is enhanced in astrocytes in active demyelinating lesions of MS [27]. The plasma osteopontin levels are elevated in active relapsing-remitting MS patients [28]. All of these observations support the concept that the selective blockade of the interaction between ECM and integrins in brain lesions *in situ* would be a target candidate for therapeutic intervention in MS.

Because focal adhesion kinase (FAK) is a central mediator of the integrin signaling pathway (see Figure 1), one possible choice is the use of an inhibitor for ECM-induced autophosphorylation of FAK [29]. TAE226, a FAK inhibitor, suppresses tumor cell invasion *in vivo* [29]. Another option for integrin signaling inhibitors is disintegrins, a group of small disulfide-rich peptides containing the arginine–glycine–aspartic acid sequence that mediates the selective binding to integrins [30]. Liposomal delivery of contortrostatin, a snake venome disintegrin, shows a tumor-suppressive anti-angiogenic activity [30]. However, a complete blockade of general function of integrins has a risk for inducing serious side effects [31]. Even in the context of the selective blockade, treatment with a humanized monoclonal antibody against VLA4, α4β1 integrin (natalizumab) reduced relapses 66% in clinical trials of MS but also activated the lethal infection of JC virus in some patients [32].