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Research article

BRAIN PROTEINS INTERACTING WITH THE TETRAMERIZATION REGION OF NON-ERYTHROID ALPHA SPECTRIN

YOUNSANG OH and LESLIE W.-M. FUNG*
Department of Chemistry, University of Illinois at Chicago, 845 W. Taylor Street, MC 111, Chicago, IL 60607, USA

Abstract: The N-terminal region of non-erythroid alpha spectrin (SpαII) is responsible for interacting with its binding partner, beta spectrin, to form functional spectrin tetramers. We used a yeast-two-hybrid system, with an N-terminal segment of alpha spectrin representing the functional tetramerization site, as a bait to screen human brain c-DNA library for proteins that interact with the alpha spectrin segment. In addition to several beta spectrin isoforms, we identified 14 proteins that interact with SpαII. Seven of the 14 were matched to 6 known proteins: Duo protein, Lysyl-tRNA synthetase, TBP associated factor 1, two isoforms (b and c) of a protein kinase A interacting protein and Zinc finger protein 333 (2 different segments). Four of the 6 proteins are located primarily in the nucleus, suggesting that spectrin plays important roles in nuclear functions. The remaining 7 proteins were unknown to the protein data base. Structural predictions show that many of the 14 proteins consist of a large portion of unstructured regions, suggesting that many of these proteins fold into a rather flexible conformation. It is interesting to note that all but 3 of the 14 proteins are predicted to consist of one to four coiled coils (amphiphilic helices). A mutation in SpαII, V22D, which interferes with the coiled coil bundling of SpαII with beta spectrin, also affects SpαII interaction with Duo protein, TBP associated

* Author for correspondence; e-mail: lfung@uic.edu, tel: (312) 355-5516, fax: (312) 996-0431

Abbreviations used: KRAB – Krueppel-associated box; QDO – quadruple drop-out; PKA – protein kinase A; SH3 – src homology 3; SpαII – non-erythroid alpha spectrin; SpI – erythroid spectrin; SpβII – spectrin beta II; TBP – TATA box binding protein; TDO – triple drop-out

factor 1 and Lysyl-tRNA synthetase, suggesting that they may compete with beta spectrin for interaction with SpαII. Future structural and functional studies of these proteins to provide interaction mechanisms will no doubt lead to a better understanding of brain physiology and pathophysiology.

Key words: Spectrin, Tetramerization site, Protein-protein interaction, Yeast-two hybrid system, Brain protein, Spectrin mutation

INTRODUCTION

Spectrin was initially characterized as a skeletal protein underneath the inner leaflet of the plasma membrane of the mature erythrocyte [1], and was known as erythroid spectrin (SpI). SpI studies have shown that several proteins, including actin, ankyrin, Band 3, protein 4.1, *etc.*, interact with spectrin tetramers, the functional form of SpI, to form a membrane skeleton [2]. In addition, structural studies have shown that the structural domains of SpI consist of three contiguous helices bundled to form a triple helical bundle [3], similar to that of *Drosophila* spectrin [4] or chicken brain spectrin [5].

Our earlier studies of spectrin tetramerization site interactions showed that the C-terminal region of beta spectrin (a helical partial domain, presumably consisting of two helices) [6], interacts with the N-terminal region of alpha spectrin (another helical partial domain, consisting of a single helix) [3], through coiled coil helical bundling [7]. With a pair of these interactions at the tetramerization site, spectrin tetramer, the functional form of spectrin, becomes the basic component of a membrane skeleton, and provides the deformability and plasticity needed for healthy erythrocytes.

At the beginning, spectrin was thought to be absent from other cell types [8]. However, spectrin is now known to be a member of the spectrin super-family, and has been found to be ubiquitous among vertebrate tissues as well as in simple metazoans, implying that spectrin isoforms play a fundamental role in cells [9-16]. Many distinct spectrin isoforms have been reported, and are believed to have evolved early in the evolution of metazoans [14], following divergence of fungi, plants and vertebrates, with each isoform representing a candidate for roles in specialized activities of multicellular animals. Spectrin is generally considered to be a structural (cytoskeletal) protein involved in stabilization of cell surface membranes at sites of cell-cell contacts [17], protein sorting [9], and protein accumulation [18]. Spectrin is also involved in regulation of signal transduction pathways [12], and in the regulation of DNA repair [19, 20]. However, studies of gene knock-outs in model organisms suggest that these genes are not essential for fundamental cellular function, but act at the level of integration of cells into tissues, and, thus, mutations may be compatible with survival but impart impaired physiological function, and are therefore candidates to cause disease in humans [14].

Currently, human spectrin isoforms include two alpha spectrin isoforms (αI & αII), and five beta spectrin isoforms (βI, βII, βIII, βIV & βH) [14]. Similar isoforms in mice have also been studied [21]. Spectrin II (SpII) is often referred to as brain spectrin, non-erythroid spectrin [22], calspectin [23] or fodrin [24], and is originally found in neuronal axons, but not dendrites [25]. SpI is confined to neuronal cell bodies and dendrites, and some glial cells, but is not present in axons or presynaptic terminals [26]. Spectrin beta III is found in brain [27] and in Golgi [28]. Spectrin isoforms have also been found in cell nuclei [29-32]. Alpha spectrin was identified at the nuclear envelope as well as in intranuclear granules within liver cells [31]. Similar localization of alpha spectrin in brain cortex sections has also been reported [32].

To understand the role of spectrin in brain physiology and pathophysiology, we have focused on molecular interactions. We have reported that several single mutations at the N-terminal region (tetramerization site) of SpαII affect its interaction with its functional partner beta II (SpβII) [32]. We suggest that such mutations may lead to neurological disorders. In this study, we identify several proteins that interact with the same N-terminal region consisting of the first 359 residues of SpαII. We have identified 7 proteins or protein fragments from known but non-beta spectrin proteins and 7 unknown proteins that interact with this functional region of SpαII. We also showed that a SpαII mutation of V22D, which diminishes the interaction with beta spectrin, affects the interaction with some of these proteins. A variety of bioinformatic searches indicate that many of the proteins identified are involved in nuclear functions.

MATERIALS AND METHODS

Materials

The MATCHMAKER GAL4 Two-Hybrid System 3, including the pGBKT7 cloning vector and two yeast strains: AH109 (*MAT***a**, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2:GAL1$_{UAS}$-*GAL1*$_{TATA}$-*HIS3, GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2, URA3:MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ MEL1*) and Y187 (*MATα, ura3-52, his3-200, Ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met*$^-$*, URA3:GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ MEL1),* was obtained from BD Biosciences Clontech (Palo Alto, CA).

A pre-transformed human brain cDNA library in the yeast strain Y187 (BD Matchmaker Library, BD Bioscience Clontech) was used. According to the manufacturer, the average insert size ranged from 0.5 kb to 3.0 kb, and the library contained at least 1 x 10^7 independent clones with < 1% genomic DNA and < 5% rRNA.

The *Pfu* turbo DNA polymerase and Quick Change Site Directed Mutagenesis Kit were from Stratagene (La Jolla, CA). T4 DNA ligase, *Eco*RI and *Bam*HI restriction endonuclease were from New England Biolabs (Beverly, MA).

Tryptone and yeast extracts were from EMD Pharmaceuticals (Durham, NC). Other culture media (YPDA, SD/-Trp, SD/-Leu, SD/-Trp/-Leu, SD/-Trp/-Leu/-His, SD/-Trp/-Leu/-His/-Ade) needed for yeast cell growth were from BD Biosciences Clontech (Mountain View, CA). X-α-gal was from LabScientific, Inc (Livingston, NJ). Synthetic oligonucleotide primers were from Operon (Huntsville, AL). The Zymoprep yeast plasmid miniprep kit was from Zymo Research (Orange, CA). Lithium acetate, semi-sulfate adenine, acrylamide, N, N-bis-acrylamide, and TEMED were from Sigma (St. Louis, MO). Other chemicals were from Fisher Scientific (Pittsburgh, PA).

Vector construction

A plasmid construct with vector designated as pBD-SpαII, which contained a cDNA fragment encoding the first 359 residues of human non-erythroid alpha spectrin (SpαII-1-359) and the GAL4 DNA-binding domain, was generated from the cloning vector pGBKT7, and used as a bait to screen the cDNA library [31]. The bait plasmid was transformed into yeast AH109 cells, using the lithium-acetate transformation method. Other plasmids, pBD-SpαII V22D, pBD-SpαII-1-147, and pBD-SpαI-1-368, were also constructed [31] and used for the yeast co-transformation experiment.

Yeast two-hybrid screening

A fresh (< 2-month old) colony of yeast cells (2-3 mm) containing pBD-SpαII vector was inoculated into synthetic dropout medium without Trp (SD/-Trp) (50 ml) and cultured at 30^{o}C for 16-24 h. Cells were harvested and re-suspended with 2 x YPDA/Kan medium (5 ml) and combined with yeast cells containing pGADT7-Rec-cDNA with > 5 x 10^7 cfu/ml (1 ml). These cells were cultured again in 2 x YPDA/Kan medium (45 ml). After 20 h mating, the diploid yeast cells were harvested, re-suspended and spread on 50 large (150 mm) plates containing triple drop-out (TDO) (SD/-His/-Leu/-Trp) medium. After 6-15 days, the yeast colonies were transferred onto plates containing quadruple drop-out (QDO) (SD/-Ade/-His/-Leu/-Trp) medium and X-α-gal to detect the MEL1 reporter gene product, α-galactosidase, which stained surviving (positive) colonies to a blue color. Plasmids from positive diploid colonies were obtained with the Zymoprep yeast plasmid miniprep kit and were further screened by PCR with 5' AD insert amplimer (5'-cta ttc gat gat gaa gat acc cca cca aac cc-3') and 3' AD insert amplimer (5'-gtg aac ttg cgg ggt ttt tca gta tct acg att-3') and *Hae*III digestion to eliminate as many identical brain cDNA inserts as possible and thus to select constructs with different inserts.

Co-transformation confirmation

Plasmids from positive diploid colonies were also transformed into *E. coli* DH5α. The *E. coli* cells, grown on plates with ampicilin to eliminate the cells with non-ampicilin resistant pBD-SpαII plasmid, were used to obtained the pGADT7-Rec-cDNA plasmid containing positive brain cDNA inserts. The

plasmids of an insert and pBD-SpαII were co-transformed into yeast strain AH109, and plated on SD/-Ade-His-Leu-Trp (QDO) plates. Plates with no protein-protein interactions had no colony growth, and those with growth indicated positive protein-protein interactions.

DNA sequencing

The positive cDNA inserts, confirmed by co-transformation experiments, were sequenced, using services provided by the Research Resource Center at the University of Illinois at Chicago.

Interaction with mutant SpαII-V22D, **SpαII-1-147, SpαI-1-368**

SpαII-V22D, a mutant found to exhibit reduced affinity with SpβII [32], was used in place of SpαII in the co-transformation experiments described above to study its interaction with the proteins identified as interacting with wild type SpαII. SpαII-1-147 and SpαI-1-368 were also co-transformed into the AH109 cells with the proteins identified from yeast two hybrid screening to study its interaction.

Gene and protein identification and characterization

Sequence data obtained for the selected cDNA library inserts of positive colonies were matched with human genes in the GenBank DNA library (National Center for Biotechnology Information), with *Blastn, Blastx and Blastp* in "Basic Local Alignment Search Tool" (http://www.ncbi.nlm. nih.gov/blast/). Each gene identified was submitted to "Bioinformatic Harvester" of the European Molecular Biology Laboratory (http://harvester.embl.de/) for human protein identification. The protein name, cellular location and structural information were extracted from the "Harvester" results. Protein sequences were also submitted to "Human Protein Reference Database" (http://www.hprd.org/) for additional functional information. For structural information, the ScanProsite tool was used on protein sequences to search for the occurrence of patterns or profiles (motifs) stored in the PROSITE database (http://www.expasy.org/ tools/scanprosite/scanprosite-doc.html). The protein sequences were also submitted to "COILS" for coiled coil (amphiphilic helical) region prediction (http://www.ch.embnet.org/software/COILS_form.html), and to "Phyre" (Protein Homology/ analogY Recognition Engine) (http://www.sbg.bio.ic.ac.uk/phyre/) for secondary structure and protein fold prediction. Predicted structural coordinates were displayed by RasMol (http://www.openrasmol.org/).

For those DNA sequences without a matching protein or gene, the most likely ORF sequences were translated using a tool from "ExPASy" (http://www.expasy.org/tools/dna.html). These protein sequences were then submitted for ScanProcite search, and COIL and Phyre predictions.

RESULTS AND DISCUSSION

Proteins interacting with SpαII

With SpαII-1-359 representing a functional tetramerization site of spectrin αII as a bait, yeast cells containing human brain cDNA inserts (1 ml, which corresponded to at least 2 x 10^6 cDNA library inserts) were screened for interactions. From the TDO/X-α-gal plates, 440 blue colonies were identified. This number was reduced to 155 with the more stringent QDO/X-α-gal plates (Fig. 1). We further eliminated 98 duplicate cDNAs following PCR and restriction enzyme digestion analysis (Fig. 2). The remaining 51 cDNA inserts were also confirmed to have positive interaction with SpαII-1-359 by the co-transformation experiments. Thus, with the highest possible stringency, we identified a total of 51 human brain cDNA inserts that interact with SpαII-1-359. Of the 51 library inserts, 37 belonged to the beta spectrin family (beta isoforms I, II, III and IV), the functional partners of SpαII, including 28 from SpβII, 6 from SpβI, 2 from SpβIII, and 1 from SpβIV (Tab. 1). The remaining 14 were non-beta spectrin proteins.

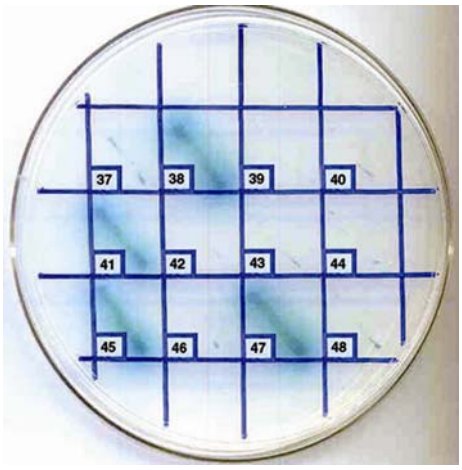

Fig. 1. Yeast two hybrid screening on QDO plates with X-α-gal. Colonies on a typical plate containing SD/-Ade/-His/-Leu/-Trp (QDO) and X-α-gal, showing white colonies at grids 37, 39, 40, 42, 43, 44, 46 and 48, and blue colonies (consisting of proteins interacting with SpαII) at grids 38, 41, 45 and 47. One hundred fifty five colonies were identified on QDO plates from 400 positive colonies obtained from SD/-His-Leu-Trp (TDO) plates, which were obtained after screening approximately 2 x 10^6 colonies.

Non-beta spectrin proteins

Seven of the 14 "non-beta spectrin" proteins matched with known proteins in the protein sequence databases used (see Materials and Methods for information on the databases) (Tab. 1). Protein 1 was a fragment (residues 181-722) of Duo

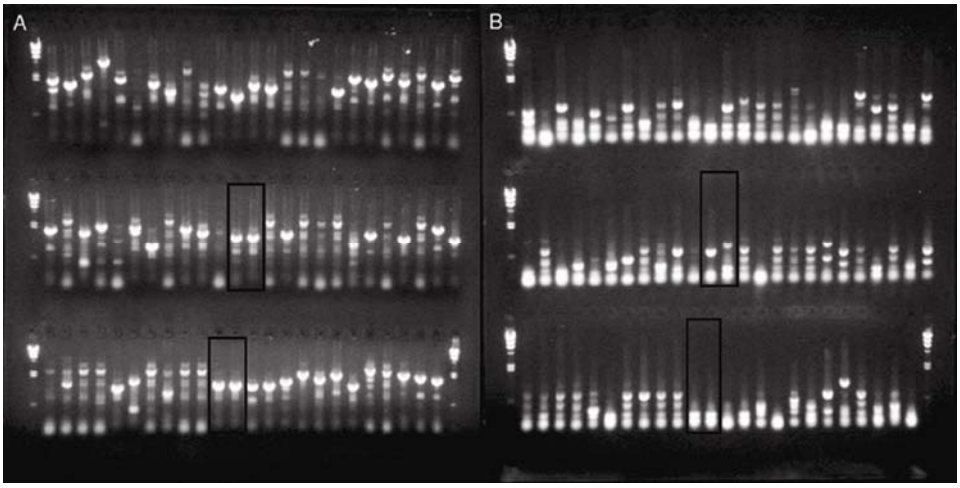

Fig. 2. PCR and restriction enzyme *HaeIII* digestion products of yeast colonies. Agarose (0.9%) gels with EtBr of representative (A) PCR products and (B) *HaeIII* digestion products of 74 yeast plasmids of positive colonies from QDO plates. For PCR products, 5' AD insert amplimer (5'-cta ttc gat gat gaa gat acc cca cca aac cc-3') and 3' AD insert amplimer (5'-gtg aac ttg cgg ggt ttt tca gta tct acg att-3') were used. The first lanes of each of the three rows of lanes were Lamda/*Hind*III DNA standard. Samples were numbered from left (second lane) to right, starting from the first row (samples 1-25), continuing to the second row (samples 26-50) and end with the third row (samples 51-74) for both (A) and (B). Samples were considered the same only when the patterns of both (the PCR product and digestion) gels of the samples appeared identical. For example, PCR products of samples 37 and 38 (A, second row, boxed lanes) appeared identical, but digestion products of samples 37 and 38 (B, second row, boxed lanes) appeared different. Thus, samples 37 and 38 were considered to be samples with different cDNA inserts, and both inserts were sequenced. Samples 61 and 62 gave similar PCR products (A, third row, boxed lanes) and digestion products (B, third row, boxed lanes), and only the cDNA insert of sample 61 was sequenced.

protein (also known as Huntingtin-associated protein-interacting protein or Kalirin). Protein 2 was a fragment (residues 1-155) of Lysyl-tRNA synthetase. Protein 3 was the full length AKIP1 (a protein kinase interacting protein 1; also known as Kyot binding protein 3) isoform b. Protein 4 was the full length isoform c. Protein 5 was a fragment (residues 1270-1495) of TBP-associated factor 1. Protein 6 was a shorter fragment (residues 1-169), and Protein 7 was a longer fragment (residues 1-230) of Zinc finger protein 333.

We were unable to match the remaining seven proteins with any known proteins in the databases. The DNA sequences of "Unnamed 1" and "Unnamed 2" genes were identified, with *blastn*, as similar to segments of genes SPATS2 and NCALD, respectively. However, no significant similarity was found with *blastx* search. The search of gene sequence for "Unnamed 3" resulted in no similar gene match. Translation of DNA sequence resulted in 77 amino acid residues for

Tab. 1. Proteins identified to interact with a recombinant peptide of SpαII consisting of the first 359 amino acid residues, including the tetramerization site of SpαII.

Colo-nies[a]	Gene[b]	Protein[c]	AA[d]	Location[e]
6	SPTB (NM_000347)	Spectrin βI	1714-2137 (2137)	
28	SPTBN1 (NM_178313)	Spectrin βII	1816-2155 (2155)	
2	SPTBN2 (NM_006946)	Spectrin βIII	1802-2390 (2390)	
1	SPTBN4 (NM_020971)	Spectrin βIV	1916-2564 (2564)	
1	KALRN (NM_001024660)	Duo protein	181-722 (2985)	C[f], 52%[g]
1	KARS (NM_005548)	Lysyl-tRNA synthetase	1-151 (597)	C, 60%
1	AKIP1b (NM_020642)	AKIP1b	1-199 (199)	N[h], 52%
1	AKIP1c (NM_020642)	AKIP1c	1-156 (156)	N, 52%
1	TAF1 (NM_138923)	TBP associated factor 1	1270-1495 (1872)	N, 91%
1	ZNF333 (NM_032433)	Zinc finger 333 (a)	1-169 (665)	N, 61%
1	ZNF333 (NM_032433)	Zinc finger 333 (b)	1-230 (665)	
1	Unnamed 1 (ES608174)	Unnamed 1	1-77	
1	Unnamed 2 (ES608175)	Unnamed 2	1-88	
1	Unnamed 3 (ES608176)	Unnamed 3	1-76	
1	Unnamed 4 (ES608177)	Unnamed 4	1-203	
1	Unnamed 5 (ES608178)	Unnamed 5	1-242	
1	Unnamed 6 (ES608179)	Unnamed 6	1-268	
1	Unnamed 7 (ES608180)	Unnamed 7	1-302	

[a]Number of colonies with the cDNA inserts whose sequences matched with the gene listed. A total of 51 library inserts were obtained from screening for interactions with SpαII; [b]From *Blastn* (see text), in parenthesis is the GenBank accession number; [c]From *Harvester* (see text); [d]Amino acid residue numbers of the start and end of the protein/protein fragment, and in the parenthesis is the number of total amino acid residues in the known protein; for spectrin beta with multiple colonies, a representative colony is used; [e]Cellular location information was obtained from Harvester; [f]C=cytoplasm; [g]Percentage of the protein in C or N; [h]N = nucleus.

"Unnamed 1" as a possible ORF sequence, 88 amino acid residues for "Unnamed 2" and 76 amino acid residues for "Unnamed 3". The gene sequences of Unnamed 4-7 were all identified as similar, but not identical, to gene FLJ40113. FLJ40113 is known as a Golgin subfamily a-like peudogene. The first 226 amino-acid residues of Unnamed 5-7 were the same, and they differed only at the C-terminal region, with Unamed 5 the shortest C-terminal region and Unnamed 7 the longest C-terminal region. The DNA sequences of these 7 unnamed proteins were submitted to GenBank and their accession number were assigned (Tab. 1).

Protein location

Beta IV is the first beta-spectrin identified to associate with a subnuclear structure and may be part of a nuclear scaffold to which gene regulatory machinery binds [33]. Out of the six known, but non-spectrin proteins identified as interacting with SpαII-1-359, four are found mostly in nucleus, with 52% of AKIP1b and AKIP1c, 91% of TBP-associated factor 1 and 61% of Zinc finger protein 333 estimated in the nucleus (Tab. 1). Duo protein and lysyl-tRNA synthetase are located primarily in the cytoplasm (52% and 60%, respectively).

Protein functional classification

Brain proteins are often categorized into five classes [34]: cell structure and motility, cell signaling and communication, nucleic acid management, protein management and metabolism (Tab. 2). Under the cell structure and motility class are beta spectrin isoforms. Duo protein is in the cell signaling and communication class, and is considered to be a guanine nucleotide exchange factor in signal transduction and vesicle trafficking [35].

Tab. 2. SpαII interacting proteins with known functions.

Functional classification	Proteins	Specific functions
Cell structure/motility	Spectrin βI	Structural protein
	Spectrin βII	
	Spectrin βIV	
	Spectrin βIV	
Cell signaling/ Communication	Duo protein	Guanine nucleotide exchange factor; signal transduction; vesicle trafficking
Nucleic acid management	Lysyl-tRNA synthetase	Lysine-tRNA Ligase; ATP & DNA binding
	TBP associa-ted factor 1	Initiation of transcription; ATP, DNA, & protein binding
	Zinc finger protein 333	Transcription factor, DNA & Zn binding, electron transporter
Protein management	AKIP1b	Binding protein kinase A, contributing to nuclear localization
	AKIP1c	
Metabolism	None	

Lysyl-tRNA synthetase, TBP-associated factor 1 and zinc finger protein 333 are in the nucleic acid management class. Lysyl-tRNA synthetase, with lysine-tRNA ligase activity, is a protein for transcription and tRNA processing and binds ATP, DNA and tRNA [36, 37]. TBP associated factor 1 is involved in initiation of transcription and has been shown to bind ATP, DNA and proteins [38-40]. Zinc finger protein 333 is a transcription factor, participating in DNA binding and zinc binding [41, 42].

AKIP1b and AKIP1c are in the protein management class. It has been reported that AKIP1 isoforms, highly conserved among mammals, are localized to the nucleus, and contribute to the integration of signaling by PKA by retaining the C subunit in the nucleus [43]. It is interesting that we did not find any proteins that could be classified as metabolic proteins (Tab. 2).

Secondary structure prediction

It is interesting that the secondary structure and protein fold prediction results (Tab. 3) showed that many of the 14 proteins that we identified consist of a large portion (an average of 44%) of unstructured regions, together with an average of 40% helical conformation and 16% sheet conformation, suggesting that most of these proteins fold into a rather flexible conformation. However, Unknown protein 4, for example, is predicted to be highly helical, whereas Unknown protein 1 is predicted to exhibit no helical conformation. More interestingly, the COIL prediction shows that all but three proteins consist of one or more coiled coils (amphiphilic helices). Actual structures obviously await experimental determination.

The search of *Prosite* for the 14 protein sequences gave only two matches of known protein structures or domain structures. They were Zinc finger protein 333 and TBP associated factor 1. TBP associated factor 1, a protein of 1,872 amino acid residues, consists of a bromodomain (residues 149-219). Bromodomain

Tab. 3. Predicted structural information of the protein segments from cDNA inserts identified to interact with the N-terminal region (tetramerization site) of SpαII.

Protein	AA[a]	2nd structural prediction[b]			Coil prediction[c]			
		H (%)	S (%)	U (%)	C1[a]	C2[a]	C3[a]	C4[a]
Duo protein	542	80	0	20	31	23		
Lysyl-tRNA synthetase	151	15	45	40	34			
AKIP1b	199	25	18	57	21			
AKIP1c	156	17	22	60	24			
TBP associated factor 1	226	51	2	47	24	13	13	
Zinc finger protein 333 (1)	169	8	18	74	13			
Zinc finger protein 333 (2)	230	19	17	64	14			
Unnamed 1	77	0	29	71	0			
Unnamed 2	88	27	15	59	0			
Unnamed 3	76	23	6	71	0			
Unnamed 4	203	96	0	4	200			
Unnamed 5	242	75	0	24	15	18	36	19
Unnamed 6	268	80	0	20	15	18	36	46
Unnamed 7	302	83	0	17	15	18	36	85

[a]Number of amino acid residues; [b]Secondary structural prediction with the program "Phyre"; H=helix; S=sheet; U=unstructured; [c]COIL prediction with the program "COIL"; C1=coil 1; C2=coil 2; C3= coil 3; C4=coil 4.

is a 75 to 110 residue protein domain, a bundle of four amphiphilic α helices, and it recognizes acetylated lysine residues at the N-terminal tails of histones. This recognition is often a prerequisite for protein-histone association and chromatin remodeling. Our TBP associated factor 1 segment consists of 226 residues near the C-terminus (residues 1270-1495) (Tab. 3).

Zinc finger protein 333 consists of the Krueppel-associated box (KRAB), a domain of about 75 amino acid residues that is found in the N-terminal part of about one third of eukaryotic Krueppel-type Cys_2His_2 zinc finger proteins and is enriched in charged amino acids [41, 44]. KRAB can be divided into sub-regions A and B, which are predicted to fold into two amphiphilic α-helices. The KRAB domain functions as a transcriptional repressor when tethered to the template DNA by a DNA-binding domain. The stretch of residues 7-74 in our Zinc finger 333 protein segment (residues1-230) is the KRAB domain.

Interaction mechanism

It has been suggested that protein interactions with dissociation constants above ~ 70 μM can be detected by a GAL4-based two-hydrid assay [45]. Therefore, the dissociation constants for the identified proteins and SpαII should be less than 70 μM.

Tab. 4. Interaction analysis of proteins with SpαII, SpαII-V22D, SpαII-1-147, or SpαI-1-368. The plasmids of ten different positive colonies were co-transformed into yeast strain AH109 with pBD-SpαII, SpαII-V22D, SpαII 1-147, or SpαI 1-368, and then plated on QDO to test the specificity of interactions. The colonies were compared after 3 weeks incubation. V22D has been reported to affect SpαII interacting with SpβII [32].

Protein	Co-transformation bait proteins			
	SpαII (1-359)	SpαII-V22D	SpαII-1-147	SpαI-1-368
Spectrin beta I	yes[a]	no[b]	yes	yes
Spectrin beta II	yes	no	yes	yes
Spectrin beta III	yes	no	not done	not done
Spectrin beta IV	yes	no	not done	not done
Duo protein (Kalirin)	yes	no	yes	no
Lysyl-tRNA synthetase	yes	no	yes	no
AKIP1c	yes	yes	yes	no
TBP-associated factor 1	yes	no	yes	no
Zinc finger protein 333	yes	yes	no	no
Unnamed protein 4	yes	yes	yes	no

[a]Colonies grew indicating interactions; [b]Colonies did not grow indicating no interactions.

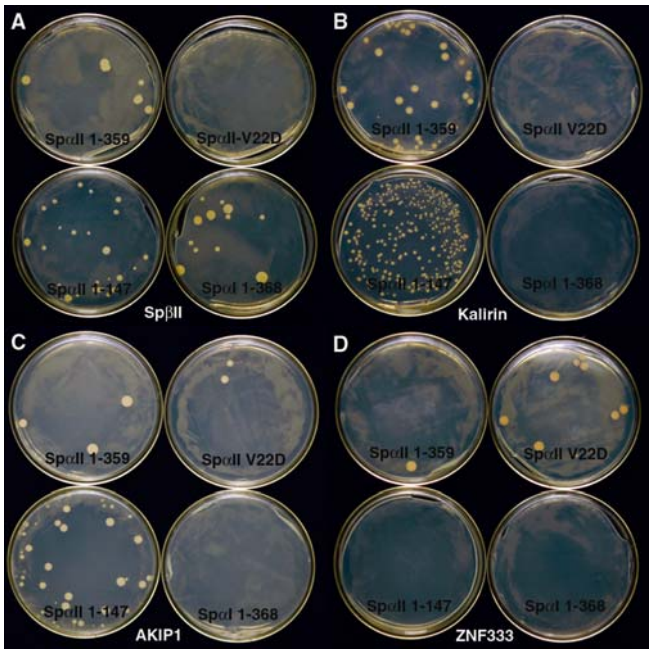

Fig. 3. Co-transformation analysis of SpαII, SpαII-V22D, SpαII-1-147 and SpαI-1-368. The positive colonies with different inserts obtained from Y2H were further analyzed by co-transformation to study the protein-protein interactions. The plasmid of a positive colony and pBD-SpαII were co-transformed into yeast strain AH109, and plated on SD/-Ade-His-Leu-Trp (QDO) plates. Panel A – the bait protein was SpβII, with SpαII-1-359 (upper left hand corner), with SpαII-V22D (upper right), with SpαI-1-368 (lower right), and with SpaII-1-147 (lower left). Panel B – the bait protein was kalirin. Panel C – the bait protein was AKIP1c. Panel D – the bait protein was Zinc finger333.

Our previous studies of spectrin tetramerization site interactions show that the C-terminal region of beta spectrin interacts with the N-terminal region of alpha spectrin, which consists of an un-associated single helix (Helix C') [3], through coiled coil helical bundling, with dissociation constants of 1.1 μM for SpαI and SpβI complex and 0.012 μM for SpαII and SpβI complex [7, 46]. Coiled coil helical bundling has been suggested to be the most common protein-protein interaction mechanism [47]. Since most proteins were predicted to form coiled coils, it is possible that these proteins interact with Helix C'of SpαII via helical bundling, in a manner similar to the alpha/beta spectrin interaction. Such a mechanism will imply possible competition of these proteins with beta spectrin in its interaction with SpαII. Replacement of amino acid residue V to D at position 22 of SpαII-1-359 resulted in little association with beta spectrin [31]. Thus, results from co-transformation experiments of SpαIIV22D were as expected in that all colonies with beta spectrin inserts did not grow (Tab. 4 and Fig. 3). Interestingly, Duo protein, lysyl-tRNA synthetase and TBP-associated

factor 1 also did not interact with SpαIIV22D. However, these proteins did interact with a shorter fragment of SpαII N-terminal end (SpαII-1-147, a protein that consists of the Helix C' partial domain followed by a single structural domain), indicating that the second and third structural domains in SpαII-1-359 are not responsible for the association with proteins identified, a finding in agreement with the suggestion that the binding sites are located on Helix C'.
The two partial domains of SpαI and SpαII exhibit 66% identical and 73% similar sequences, yet the erythroid fragment of SpαI-1-368 did not interact with the proteins identified, indicating that the interactions of the proteins with SpαII-1-359, or with SpαII-1-147, are specific to SpαII.

Suggested functions in nuclei

Spectrin isoforms have been found in cell nuclei [28-31]. Spectrin is involved in the dynamics of nuclear architecture during mitosis [12]. Alpha spectrin is identified at the nuclear envelope [30]. More recently, Lambert and colleagues demonstrated that SpαII is involved in DNA repair in the nucleus [19, 20, 48, 49] (Tab. 2). Indeed, SpαII was described as a component of a chromatin- associated complex that was involved in the repair of DNA inter-strand crosslinks, and was identified as being a direct binding partner of the DNA-repair proteins, FANCA and FANCC [48]. Subsequent studies have shown that SpαII binds directly to DNA inter-strand cross-links [18, 49]. SpαII is localized to multiple nuclear foci along with two DNA-repair proteins (FANCA and XPF) to which it binds directly [49]. Recent studies suggested that SpαII may play a role in a number of diverse and important processes in the nucleus [20].
It has also been reported that erythroid protein 4.1 (4.1R), originally identified as a component of the membrane-skeleton of the erythrocyte, could play an important role in organizing the nuclear architecture, mitotic spindle, and spindle poles [50-52]. Protein 4.1N is a neuronal selective isoform of protein 4.1R, and appears to mediate the anti-proliferative actions of NGF by antagonizing the role of NuMA in mitosis [53]. Thus, a network of spectrin, actin, and protein 4.1, similar to that found in the erythrocyte, may localize in the nucleus and solicit other functionally important proteins to provide critical functions for the nucleus.
Our finding of Duo protein, Lysyl-tRNA synthetase, TBP-associated factor 1, AKIP1b and c, and zinc finger protein 333 interacting with the alpha spectrin tetramerization site is compatible with the current view of compartmentalization and dynamic reorganization in the nucleus [54-56]. In addition, proteins with currently unknown functions also interact with the SpαII tetramerization site, suggesting additional functions for alpha spectrin in the brain. Future structural and functional studies of these proteins, including the unknown proteins, to provide interaction mechanisms will no doubt lead to a better understanding of brain physiology and pathophysiology.

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