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Research article

NON-ERYTHROID BETA SPECTRIN INTERACTING PROTEINS AND THEIR EFFECTS ON SPECTRIN TETRAMERIZATION

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Abstract: With yeast two-hybrid methods, we used a C-terminal fragment (residues 1697-2145) of non-erythroid beta spectrin (βII-C), including the region involved in the association with alpha spectrin to form tetramers, as the bait to screen a human brain cDNA library to identify proteins interacting with βII-C. We applied stringent selection steps to eliminate false positives and identified 17 proteins that interacted with βII-C ($IP_{\beta II\text{-}C}$ s). The proteins include a fragment (residues 38-284) of "THAP domain containing, apoptosis associated protein 3, isoform CRA g", "glioma tumor suppressor candidate region gene 2" (residues 1-478), a fragment (residues 74-442) of septin 8 isoform c, a fragment (residues 704-953) of "coatomer protein complex, subunit beta 1, a fragment (residues 146-614) of zinc-finger protein 251, and a fragment (residues 284-435) of syntaxin binding protein 1. We used yeast three-hybrid system to determine the effects of these βII-C interacting proteins as well as of 7 proteins previously identified to interact with the tetramerization region of non-erythroid alpha spectrin ($IP_{\alpha II\text{-}N}$ s) [1] on spectrin tetramer formation. The results showed that 3 $IP_{\beta II\text{-}C}$ s were able to bind βII-C even in the presence of αII-N, and 4 $IP_{\alpha II\text{-}N}$ s were able to bind αII-N in the presence of βII-C. We also found that the syntaxin

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Abbreviations used: αII - non-erythroid (brain) alpha spectrin; αII-N - a recombinant protein consisting of the N-terminal region 359 residues of αII; AD - activation domain of GAL4; βII - non-erythroid (brain) beta spectrin; βII-C - a recombinant protein consisting of residues 1697-2145 at the C-terminus of βII; BD - binding domain of GAL4; $IP_{\alpha II\text{-}N}$ - proteins interacting with αII-N; $IP_{\beta II\text{-}C}$ - proteins interacting with βII-C; pAD - yeast two-hybrid cloning vector pGADT7; pBD - yeast two-hybrid cloning vector pGBKT7; pBR - yeast three-hybrid cloning vector pBridge; QDO - quadruple drop-out; SD - synthetic defined; TDO - triple drop-out; X-α-gal - 5-bromo-4-chloro-3-indolyl-α-galacto-pyranoside; YPDA - yeast growth medium with yeast extract, peptone, dextrose and adenine

binding protein 1 fragment abolished αII-N and βII-C interaction, suggesting that this protein may inhibit or regulate non-erythroid spectrin tetramer formation.

Key words: Brain beta spectrin, Spectrin tetramerization, Brain proteins, Yeast three-hybrid, Library screening, Spectrin interacting proteins

INTRODUCTION

Spectrin is a cytoskeletal protein, initially identified for its role in preserving the biconcave shape of erythrocyte membranes [2] and originally considered to be present only in erythrocyte [3] until the identification of non-erythrocyte isoforms and their functions [4]. Spectrin is involved in the formation and maintenance of plasma membranes at sites of cell-cell contacts [5], protein sorting and accumulation [6], interactions with structural and regulatory proteins [7], regulation of signal transduction pathways [8], and regulation of DNA repair [9]. Non-erythroid spectrin (spectrin II), also referred to as brain spectrin [10], calspectin [11], or fodrin [12], is found in neuronal axons [13], whereas erythroid spectrin (spectrin I) is confined to neuronal cell bodies and dendrites, and some glial cells [14]. Beta II spectrin (βII) is found in cell bodies as well as axons (*e.g.* [15]). Furthermore, βI spectrin is absent from Purkinje cell dendrites [16]. βII participates in the propagation of TGF-β signaling [17]. Gene knock-out studies show that spectrin expression and regulation are important for fundamental cellular functions. Many spectrin mutations are non-lethal but cause disease conditions in humans [18]. Studies have also shown that knockdown of αII spectrin is lethal, and spectrin is an essential protein in the cell [5, 19, 20]. Spectrin tetramer is the functional form in many cells [21, 22], and, for example, spectrin tetramer is important in neuritogenesis [22].

Spectrin tetramerization involves interaction of the lone helix (Helix C') at the N-terminal region of α-spectrin of one αβ heterodimer and the two helices (Helix A' and Helix B') at the C-terminal region of the β-spectrin on another heterodimer [23-26]. This interaction involves hydrophobic residue clustering, salt bridges and hydrogen bonds [25-29]. Despite high sequence homology and three-dimensional structural similarity, dissociation constant measurements using model proteins of different spectrin fragments show two orders of magnitude difference in the N-terminal α-spectrin and C-terminal β-spectrin association affinity between erythroid and non-erythroid spectrin [30, 31], in good agreement with earlier studies using intact spectrin [32]. It has been shown that other proteins also interact with the N-terminal region of αII-spectrin [1]. They include Duo protein, Lysyl-tRNA synthetase, TBP associated factor 1, two isoforms (b and c) of a protein kinase A interacting protein and 2 different segments of Zinc finger protein 333 as well as several unknown proteins. These proteins may compete with its spectrin partner to regulate spectrin tetramerization and cytoskeletal structures.

In this study, we identified seventeen proteins that interact with a recombinant protein consisting of the C-terminal tetramerization site of βII-spectrin (βII-C). The proteins include a fragment (residues 38-284) of "THAP domain containing, apoptosis associated protein 3, isoform CRA g", "glioma tumor suppressor candidate region gene 2", a fragment (residues 74-442) of septin 8 isoform c, a fragment (residues 704-953) of "coatomer protein complex, subunit beta 1", a fragment (residues 146-614) of zinc-finger protein 251, and a fragment (residues 284-435) of syntaxin binding protein 1. These 17 proteins, along with 7 proteins that interact with the N-terminal region of αII-spectrin (αII-N) mentioned above, have been tested for their effects on spectrin tetramerization. One βII-C interacting protein abolishes αII-N and βII-C interaction. This protein of 153 residues, except the last 8 residues, is identical to a fragment (residues 284-428) of syntaxin binding protein 1. We also studied the effects of these proteins on αII-N and βII-C association and found that the binding of syntaxin binding protein 1 fragment to βII-C abolishes the αII-N and βII-C association, suggesting that this protein may inhibit or regulate non-erythroid spectrin tetramerization.

MATERIALS AND METHODS

Library screening for βII-C interacting proteins ($IP_{βII-C}$)

The C-terminal region (amino acid residues 1697-2145) of brain (non-erythroid) beta spectrin (accession number Q01082) (βII-C) was used as the bait to screen for interacting proteins in the human brain cDNA library (BD Matchmaker Library, BD Biosciences Clontech). The sequence encoding βII-C was cloned to the binding domain (BD) plasmid (pBD) using standard methods [1, 33], and labeled as pBD-βII-C.

To test for potential toxic effects of BD-βII-C fusion protein, AH109 cells were co-transformed with pBD-βII-C and an empty activation domain (AD) plasmid (pAD). Briefly, several colonies of AH109 cells were grown in medium with yeast extract, peptone, dextrose, and adenine (YPDA, 50 ml) at 30°C overnight, before transferring to a fresh YPDA with kanamycin (300 ml) until an OD_{600} of 0.6 (about 3 hours), following procedures in the user manual. Cells were harvested, washed with tris-EDTA solution and suspended in tris-EDTA plus lithium acetate solution (see user manual for solution preparation). pBD-βII-C and pAD plasmids (0.1 μg of each), and Herring Testes carrier DNA (0.1 mg) were mixed with the cell suspension (100 μl). Polyethylene glycol and lithium acetate solution (600 μl) was added and the mixture was incubated at 30°C for 30 min. Dimethyl sulfoxide (70 μl) was added, before a heat shock step at 42°C for 15 min. Cells were briefly centrifuged, re-suspended in sterile tris-EDTA solution before spreading on agar plates containing synthetic defined (SD) minimal medium with double drop-out (DDO, SD/-Leu/-Trp) supplement and grown for 3 days at 30°C.

To test for potential non-specific activation of the reporter genes giving false positive results in screening, AH109 cells with pBD-βII-C and an empty pAD plasmids were spread on agar plates containing SD minimal medium supplemented with quadruple drop-out (QDO, SD/-Ade/-His/-Leu/-Trp) and grown for 3 days at 30°C.

For library screening, bait plasmid pBD-βII-C was transformed into yeast strain AH109. A freshly transformed colony, 2-3 mm in size, was inoculated into SD medium with drop-out supplement lacking tryptophane (SD/-Trp, 50 ml) and grown until cells reached stationary phase (OD_{600} > 1.5). AH109 cells were harvested, re-suspended with a "2X YPDA" plus kanamycin solution (5 ml) and mated with Y187 cells containing library plasmids (pAD-$IP_{\beta II\text{-}C}$) with > 5 x 10^7 cfu/ml (1 ml). These cells were cultured again in 2X YPDA with kanamycin (45 ml) for 20 hours at 30°C with slow shaking (30-50 rpm). Diploid cells were collected and spread on 50 large (150 mm) plates containing SD medium with QDO supplement and grown for 5 days at 30°C. Well isolated colonies growing on these plates were selected, and ones showing coalescent growth were avoided.

Further selection to obtain positive colonies was done by transferring selected colonies to QDO plates with the chromagenic substance, X-α-gal, and grown for 3 days at 30°C. Those colonies with α-galactosidase production were detected by the appearance of blue colonies as they grew on plates.

Co-transformation for confirmation of screened interacting proteins

Plasmids purified from positive colonies were transformed into *E. coli* DH5α cells, using conventional methods. Cells that were able to grow on plates with ampicillin were used to eliminate kanamycin resistant pBD-βII-C plasmid, and to obtain pAD-$IP_{\beta II\text{-}C}$ plasmids in positive colonies. Purified pAD-$IP_{\beta II\text{-}C}$ and pBD-βII-C plasmids were co-transformed into the AH109 cells and plated on QDO plates. After 3 days at 30°C, cells without growth were eliminated, and only those with growth were further analyzed for $IP_{\beta II\text{-}C}$ s.

Effects of interacting proteins on spectrin tetramerization

We also identified the $IP_{\beta II\text{-}C}$ s that were able to bind to βII-C in the presence of αII-N (first 359 residues in αII) by using the yeast three-hybrid vector, pBridge (pBR), to express not only the binding domain fusion protein, BD-βII-C, but also to express an additional protein (such as αII-N) only in the absence of methionine in the growth medium. In the presence of methionine, this additional protein was not expressed, and thus can be used as a control sample. AH109 cells were co-transformed with pBR-βII-C--αII-N and pAD-$IP_{\beta II\text{-}C}$ plasmids. These cells were plated on agar plates containing SD medium with TDO supplement in the absence of methionine in the growth medium to express BD-βII-C and AD-$IP_{\beta II\text{-}C}$ as well as αII-N, and in the presence of methionine to express only BD-βII-C and AD-$IP_{\beta II\text{-}C}$, and allowed to grow for 3 days at 30°C.

Once the $IP_{\beta II-C}$ s that were able to bind to βII-C in the presence of αII-N were selected, we then selected those that abolish the interaction between βII-C and αII-N by using AH109 cells co-transformed with pBR-αII-N--$IP_{\beta II-C}$ and pAD-βII-C plasmids to express BD-αII-N and AD-βII-C as well as $IP_{\beta II-C}$, in the absence of methionine.

In addition to $IP_{\beta II-C}$ s from this screening, we also studied the effect of αII-N interacting proteins ($IP_{\alpha II-N}$ s) identified in our earlier screening [1], on αII-N and βII-C interaction by using a similar experimental set up, replacing $IP_{\beta II-C}$ with $IP_{\alpha II-N}$ in the plasmids used. The seven proteins used were Zinc finger protein 333 - fragment 1-169, Zinc finger protein 333 - fragment 1-230, AKIP1b, lysyl-tRNA synthetase - fragment 1-151, TBP associated factor 1-fragment 1270-1495, Duo protein - fragment 181-722 and spectrin βIV - fragment 1916-2564 [1].

DNA sequencing and protein identification

Plasmids from positive colonies with $IP_{\beta II-C}$ s were sequenced at the DNA Services Facility, Research Resources Center at the University of Illinois at Chicago. Sequencing results were analyzed with Clustal W v1.7 (EMBL, Heidelberg, DE) to identify the sequences of SMART III, CDSIII, and poly A tail in each plasmid, and the segment between the SMARTIII and CDSIII sequences was marked as the sequence for the library cDNA. Since the sequence of SMARTIII may vary in pAD-$IP_{\beta II-C}$ plasmids (see Clontech manual for SMART cDNA library construction), all three possible reading frames of DNA sequence were examined (Frame 0, following the Clontech codon assignment for SMARTIII for the rest of the plasmid; Frame +1, frame with one additional nucleotide; Frame -1, frame with two additional nucleotides). The frame containing the most codons before the first stop codon was selected. Amino acid sequences were determined using the Translate tool (ExPASy proteomics server), and the sequences between SMARTIII and the first stop codon was taken as the sequence for the interacting protein. These sequences were analyzed using Blastn, Blastx, and Blastp in "Basic Local Alignment Tool" (http://www.ncbi.nlm.nih.gov/blast/) in all non-redundant BLAST protein sequence databases, as before [1], for information on the interacting proteins.

RESULTS

Library screening for βII-C interacting proteins

Tests for toxic effects of βII-C on yeast growth showed several colonies, 2-5 mm in diameter (Fig. 1A), indicating that βII-C is not toxic to the yeast cells. In the test for false positive in screening, yeast cells with pBD-βII-C and empty pAD plasmids did not form any colonies (Fig. 1B), indicating that colony growth is observed only in the presence of an interacting protein X expressed by the pAD-X plasmid.

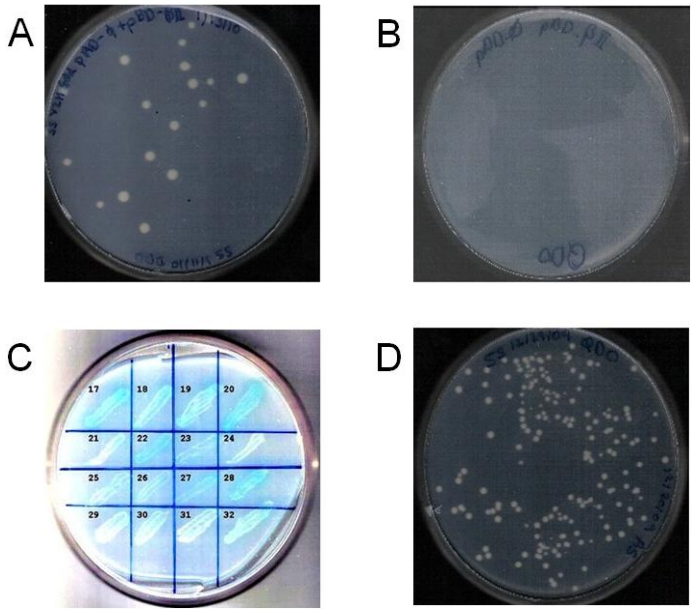

Fig. 1. Library screening for βII-C interacting proteins. AH109 cell colonies with pBD-βII-C and pAD plasmids supplemented with Ade and His (DDO medium) after 3 days of growth at 30°C, indicating that pBD-βII-C is not toxic to yeast cell growth (A). Same cells without Ade and His supplement (QDO medium) after 3 days of growth at 30°C, shows no colonies indicating that pBD-βII-C does not lead to the false activation of the reporter genes (B). Colonies from screening transferred to QDO plates with X-α-gal, and grown for 3 days at 30°C, where 59 colonies, such as on grids 17-20, 22-23, 25-28 and 32, turned blue, while most of the colonies, such as on grids 21, 24 and 29-31, grew as white colonies (C). AH109 cells with one of the 20 randomly selected sequences (pAD-$IP_{βII-C}$-1) and pBD-βII-C plasmids on QDO plates, after 3 days of growth at 30°C, shows high numbers of colonies, confirming the presence of positive interactions between βII-C and $IP_{βII-C}$-1 (D).

In the library screening experiments for identifying βII-C interacting proteins, we selected 299 well separated colonies and avoided coalescent colonies. We further selected only those colonies (a total of 59) that produced α-galactosidase to give blue colonies (Fig. 1C, for example, colonies on grids 17-20, 22-23, 25-28 and 32) and eliminated 240 of those colonies that appeared as white colonies (Fig. 1C, colonies on grids 21, 24 and 29-31) and were considered to be false positives. In the co-transformation confirmation analysis, randomly selected 20 of the 59 blue colonies showed colony growth in all 20 samples (Fig. 1D), confirming that these colonies indeed consisted of proteins that interacted with βII-C.

DNA sequencing results of the βII-C interacting proteins

Sequence analysis of the cDNA sequences between SMARTIII and CDSIII of the 20 confirmed plasmids revealed that the sequences of the cDNA fragments ranged from 487 to 1,744 nucleotides. Three of the cDNA sequences were

identical to each other, and another 2 of the cDNA sequences were also identical to each other (Tab. 1). Thus, a total of 17 different sequences were obtained from the 20 randomly selected positive colonies (Tab. 1). Selecting the largest number of codons from one of the three frames (-1, 0 and +1 frames, see Methods), 8 of the 17 sequences were with frame 0, with the SMARTIII sequence ending as ATG GCC (Tab. 1, #1-5 and 7-9). For #6 only 49 codons were obtained for frame 0, but 303 codons for frame +1, suggesting that the SMARTIII sequence for this sample ended with one extra nucleotide (**G** in TTA TGG CC**G**). A similar frame shift was observed for #11 and 17.

Tab. 1. DNA sequencing analysis of the library plasmids that show positive interactions with the C-terminal region (residues 1697-2145) of non-erythroid β spectrin (βII-C).

$IP_{\beta II-C}$	Nucleotides[a]	Frame[b]	Codons[c]	First three residues[d]	Occurrence
1	1536	0	**153** (6, 30)	DDD	3
2	1509	0	**483** (0, 4)	SSF	1
3	1509	0	**483** (0, 4)	SSF	1
4	1744	0	**278** (10, 39)	RVG	1
5	1325	0	**386** (10, 39)	RVG	1
6	1400	1	303 (7, **49**)	KKK	2
7	1232	0	**247** (42, 108)	GGS	1
8	602	0	**131** (16, 24)	GGR	1
9	1026	0	**250** (5, 8)	EAA	1
10	784	-1	260 (**21**, 30)	ELG	1
11	1678	1	124 (73, **40**)	LGK	1
12	1484	-1	369 (**4,** 26)	ASH	1
13	487	-1	54 (**16**, 3)	GEV	1
14	1121	-1	84 (**14**, 28)	PQP	1
15	1083	-1	26 (7, 15)	ERE	1
16	889	-1	18 (**10**, 3)	QAW	1
17	1672	1	78 (39, **27**)	ILP	1

[a]Number of nucleotides between SMARTIII and CDSIII; [b]Frame 0 uses the codon assignment by Clontech for SMARTIII sequence; Frame +1 is with one extra nucleotide; Frame -1 is with two extra nucleotides; [c]Number of codons for the assigned frame, with those for other frames given in parentheses and the numbers for Frame 0 bolded; [d]first three amino acid residues in the protein.

For #10, only 21 codons were obtained for frame 0, 30 codons for frame +1, but 260 codons for frame -1, suggesting that the SMARTIII sequence for this sample ends with two extra nucleotides (**GG** in TAT GGC C**GG**). Similar frame shift was observed for #12-16. The first three amino acid residues of each translated proteins are shown in Tab. 1 for identification references.

αII-N effect on $IP_{βII-C}$ interaction with βII-C

The yeast three-hybrid experiments with the cells of the 17 samples grown in the presence of methionine to express AD-$IP_{βII-C}$ and BD-βII-C, but not αII-N show colony growth (data not shown), as expected, confirming the interactions between AD-$IP_{βII-C}$ and BD-βII-C in these cells. However, for cells grown in the absence of methionine leading to the expression of αII-N alongside AD-$IP_{βII-C}$ and BD-βII-C, 14 samples showed no colony formation, indicating these $IP_{βII-C}$ s did not interact with βII-C in the presence of αII-N. Only the cells with three $IP_{βII-C}$ s ($IP_{βII-C}$-1, -8 and -9) gave colonies (Tab. 2A), indicating that $IP_{βII-C}$-1, -8 and -9 interacted with βII-C in the presence of αII-N. Of these three $IP_{βII-C}$ s that interact with βII-C in the presence of αII-N, we expressed $IP_{βII-C}$-1 in cells with BD-αII-N and AD-βII-C (pBR-αII-N--$IP_{βII-C}$ and pAD-βII-C plasmids, in the absence of methionine) and found no colony growth (Tab. 2C), indicating that the presence of $IP_{βII-C}$-1 abolished the αII-N and βII-C interaction. In the presence of methionine, when no $IP_{βII-C}$ -1 was expressed, colonies formed (data not shown).

Tab. 2. Results of yeast three-hybrid experiments for (A) effects of αII-N on βII-C and $IP_{βII-C}$ interaction (B) effects of βII-C on αII-N and $IP_{αII-N}$ interaction, and (C) effect of $IP_{βII-C}$ on αII-N and βII-C interaction.

A. Cells with plasmids pAD-$IP_{βII-C}$ and pBR-βII-C--αII-N were grown in the absence of methionine to express AD-$IP_{βII-C}$, BD-βII-C and αII-N.

$IP_{βII-C}$	$IP_{βII-C}$-1	$IP_{βII-C}$-2	$IP_{βII-C}$-3	$IP_{βII-C}$-4	$IP_{βII-C}$-5	$IP_{βII-C}$-6	$IP_{βII-C}$-7	$IP_{βII-C}$-8	$IP_{βII-C}$-9	$IP_{βII-C}$-10	$IP_{βII-C}$-11	$IP_{βII-C}$-12	$IP_{βII-C}$-13	$IP_{βII-C}$-14	$IP_{βII-C}$-15	$IP_{βII-C}$-16	$IP_{βII-C}$-17
Colony growth	yes	no	no	no	no	no	no	yes	yes	no	no	no	no	no	no	no	no

B. Cells with plasmids pAD-$IP_{αII-N}$, and pBR-αII-N--βII-C were grown in the absence of methionine to express AD-$IP_{αII-N}$, BD-αII-N and βII-C.

$IP_{αII-N}$	$IP_{αII-N}$-1	$IP_{αII-N}$-2	$IP_{αII-N}$-3	$IP_{αII-N}$-4	$IP_{αII-N}$-5	$IP_{αII-N}$-6	$IP_{αII-N}$-7
Colony growth	yes	yes	no	yes	yes	no	no

C. Cells with plasmids pAD-βII-C and pBR-αII-N--$IP_{βII-C}$-1, or pBR-αII-N--$IP_{αII-N}$-5, were grown in the absence of methionine to express AD-βII-C, BD-αII-N and $IP_{βII-C}$-1 or $IP_{αII-N}$-5.

IP	$IP_{βII-C}$-1	$IP_{αII-N}$-5
Colony growth	no	yes

Alignment of βII-C interacting protein sequences to human proteins

The sequence alignment results of the 17 $IP_{\beta II\text{-}C}$ sequences to protein sequences in the database show that only $IP_{\beta II\text{-}C}$-7, except the last 8 residues, is identical to a fragment (residues 38-284) of a known protein -- "THAP domain containing, apoptosis associated protein 3, isoform CRA g" (Tab. 3). Four of the proteins were 99% identical to known proteins or protein fragments, including glioma tumor suppressor candidate region gene 2 (residues 1-478), septin 8 isoform c (residues 74-442), and coatomer protein complex, subunit beta 1 (residues 704-953) (Tab. 3). We are puzzled about the identity of $IP_{\beta II\text{-}C}$-10, which is identical to βII spectrin, residues 1781-2040 except with two mutations (Tab. 3). $IP_{\beta II\text{-}C}$-4 was similar to zinc-finger protein 251 (residues 146-614) (2% difference), and $IP_{\beta II\text{-}C}$-1

Tab. 3. βII-C interacting proteins ($IP_{\beta II\text{-}C}$ s) and their effects on tetramerization site interaction.

$IP_{\beta II\text{-}C}$	Matching sequence	Homo sapiens proteins in databases (accession #), matching fragment	Difference	αII-N[a]	Effect on tetramer[b]
7	1-247 (247)[c]	THAP domain containing, apoptosis associated protein 3, isoform CRA g (EAW71568), residues 38-284 (284)[c]	0%	yes	no
10	1-260*[d] (260)	Spectrin, beta, non-erythroid (Q01082), residues 1781-2040 (2314)	1% *G20E, F235S[d]	yes	no
2	6-483 (483)	Glioma tumor suppressor candidate region gene 2 (NP_056525), residues 1-478 (478)	1% first 5 aa (SSFDK)	yes	no
12	1-369* (369)	Septin 8 isoform c (NP_001092282), residues 74-442 (442)	1% *H239Q, P344T, F362S	yes	no
9	1-250* (250)	Coatomer protein complex, subunit beta 1 (NP_057535), residues 704-953 (953)	1% *Y53C	no	not tested
4	1-271 (278)	Zinc-finger protein 251 (NP_612376), residues 146-416 (671)	2% last 7 residues	yes	no
1	1-145 (153)	Syntaxin binding protein 1 (NP_003156), residues 284-428 (603)	4% last 8 residues	no	yes
3	6-263, 288-483 (483)	Glioma tumor suppressor candidate region gene 2 (NP_056525), residues 1-258; 283-478 (478)	6% first 5 aa (SSFDK)	yes	no
8	19-131 (131)	Ubiquitin-conjugating enzyme E2L3 (BAG61806), residues 100-212 (212)	14% first 18 aa	no	not tested
6	1-247*[c] (303)	Golgin A6 family-like 10 (NP_001157937), (479)	26%	yes	no
5	32-386* (386)	Zinc-finger protein 251 (NP_612376), residues 317-671 (671)	27% (*numerous mutations)	yes	no
11	4-79 (124)	Eukaryotic translation initiation factor 3, subunit H (EAW91959), residues 1-76 (332)	30% (*numerous mutations)	yes	no
13-17		Unknowns 1, 2, 3, 4		yes	no

[a]effects of αII-N on $IP_{\beta II\text{-}C}$ and βII-C interaction, with "yes" indicating that αII-N abolishes $IP_{\beta II\text{-}C}$ interaction with βII-C; [b]effects of $IP_{\beta II\text{-}C}$ on αII-N and βII-C interaction; [c]total number of residues in protein; [d]the symbol * indicates mutations exist. [e]$IP_{\beta II\text{-}C}$ res 1-150 correspond to res 47-196 with 5 mutations (I4V, E39Q, R156H, R163C, Q169L, D174E, R191C), res 151-168 correspond to res 211-228 and res 169-247 correspond to res 243-321 with E237D.

was similar to syntaxin binding protein 1 (residues 284-435) (4% difference). The remaining proteins exhibited lower homology values to known proteins (Tab. 3). Those proteins identified that deviate from the human prototype standards, even by a single amino acid, should be considered as a "new" protein. The mutation(s) may or may not affect the interactions with the bait protein. For example, $IP_{\beta II\text{-}C}$-4 was similar to zinc-finger protein 251 fragment (residues 146-614), but with 2% difference. Our results provide no information on whether zinc-finger protein 251 fragment (residues 146-614) interacts with βII-C, unless verified by additional analysis using the fragment of zinc-finger protein 251 (residues 146-614). Thus, some of the interactions reported here may represent artifactual positives based on mutant sequences.

βII-C effect on $IP_{\alpha II\text{-}N}$ interaction with αII-N

For the 7 αII-N interactors identified previously (see Methods), cells grown in the presence of methionine, consisting of AD-$IP_{\alpha II\text{-}N}$ and BD-αII-N, but no βII-C, showed colony growth, confirming the interactions between AD-$IP_{\alpha II\text{-}N}$ and BD-αII-N in these cells (data not shown). Four of the 7 samples of cells grown in the absence of methionine, with βII-C being expressed along side with AD-$IP_{\alpha II\text{-}N}$ and BD-αII-N, gave colonies, indicating that $IP_{\alpha II\text{-}N}$-1 (TBP-associated factor), $IP_{\alpha II\text{-}N}$-2 (lysyl-tRNA synthetase), and $IP_{\alpha II\text{-}N}$-4 and $IP_{\alpha II\text{-}N}$-5 (two fragments of Zinc finger protein 333, 1-169 residues and 1-230 residues fragments, respectively) interacted with αII-N in the presence of βII-C. Cells with $IP_{\alpha II\text{-}N}$-3, $IP_{\alpha II\text{-}N}$-6 and $IP_{\alpha II\text{-}N}$-7 did not show any colony growth (Tab. 2B).

Of the 4 $IP_{\alpha II\text{-}N}$ s that interact with βII-C in the presence of αII-N, Zinc finger protein 333 - fragment 1-230 was selected to test for its effect on αII-N and βII-C interactions. In the absence of methionine, with the presence of Zinc finger protein 333 - fragment 1-230, colony growth was observed, indicating that Zinc finger protein 333 - fragment 1-230 did not abolish the αII-N and βII-C interaction (Tab. 2C).

DISCUSSION

Tetramerization is an important process for spectrin isoforms, and involves helical bundling of three helices, one from the α- and two from the β-spectrin [14, 25, 28]. The bundled complexes exhibit different K_d values, with the non-erythroid complex αII-N/βII-C about 10 nM and the erythroid complex about 1 μM [31]. Proteins have been identified that interact with αII spectrin at the tetramerization site, and we suggest that these proteins may regulate the affinity between αII-N and βII-C [1]. In this study, we identified 17 proteins that interacted with βII spectrin at the tetramerization site. Eight of these 17 proteins were very similar to existing proteins, with one ($IP_{\beta II\text{-}C}$-7) identical to "THAP domain containing, apoptosis associated protein 3, isoform CRA g". Each member of the THAP family consists of a conserved domain [34], the THAP domain, which is a putative DNA-binding domain and probably also binds a zinc

ion. This is a novel protein motif with similarity to the DNA-binding domain of P element transposase in *Drosophila* [35]. Another βII-C interactor ($IP_{βII-C}$-4) also binds zinc ion and is identical to a fragment (residues 146-416) of zinc finger protein 251 (ID#: Q9BRH9), with an additional 7 residues at the C-terminus. It is interesting to note that fragments (residues 1-169 and 1-230) of zinc finger protein 333 are αII-N interacting proteins [1]. And, in this study, we found that these fragments associate with αII-N even in the presence of βII-C, but it did not abolish the αII-N and βII-C association. Similarly, the zinc finger protein 251 fragment associates with βII-C even in the presence of αII-N, but it did not abolish the αII-N and βII-C association in tetramer formation. $IP_{βII-C}$-5 is also similar to zinc finger protein 251 (residues 317-671), but with numerous mutations.

$IP_{βII-C}$-2, other than the first 5 residues, is identical to "glioma tumor suppressor candidate region gene 2", in its entirety. It is also interesting that we identified another protein ($IP_{βII-C}$-3) that consists of the first 258 residues and residues 283-478 of this protein. $IP_{βII-C}$-12 is identical to a C-terminal fragment (residues 74-442) of septin 8 isoform c, except with three mutations (H239Q, P344T and F362S). Septin 8 isoform c is a member of the large septin family that performs diverse cellular functions according to tissue expression and their interacting partners. Functions include cell division, chromosome segregation, protein scaffolding, cellular polarity, motility, membrane dynamics, vesicle trafficking, exocytosis, apoptosis, and DNA damage response [36, 37]. The 6 $IP_{βII-C}$ s discussed above did not interact with βII-C in the presence of αII-C, suggesting that their affinities with βII-C are weaker than that of αII-N with βII-C.

$IP_{βII-C}$-9, identical to the C-terminal 250 residues of coatomer subunit beta (residues 704-953) except with one mutation (Y to C, residue Y756 in coatomer subunit beta and residue C53 in $IP_{βII-C}$-9), interacts with βII-C even in the presence of αII-N, suggesting strong affinity with βII-C. Similar to $IP_{βII-C}$-9 in affinity are $IP_{βII-C}$-1 and $IP_{βII-C}$-8. $IP_{βII-C}$-1, except for the last 8 residues, is identical to a fragment (residues 284-428) of syntaxin binding protein 1. Syntaxin binding protein 1 appears to play a role in the release of neurotransmitters via regulation of syntaxin, a transmembrane attachment protein receptor [38]. $IP_{βII-C}$-8, except the first 18 residues, is identical to ubiquitin-conjugating enzyme E2L3, residues 100-212. This enzyme participates in the ubiquitination of p53, c-Fos and the NF-κB precursor p105 in vitro [39, 40]. With these three strongly interacting proteins, only $IP_{βII-C}$-1, a 153-residue protein and its residues 1-145, which are identical to residues 284-428 of syntaxin binding protein 1, abolished αII-N and βII-C interaction.

Until demonstrated by future experimental results, it is also possible that the interactions between specific $IP_{βII-C}$ and βII-spectrin may not regulate spectrin tetramer formation. It is possible that these interactions may play a role in other cellular processes. As indicated in a recent review, the spatial and temporal organization of molecules within a cell is critical for coordinating the many

distinct activities carried out by the cell [41]. Scaffold proteins, including actin-spectrin cytoskeleton, have been found to play a central role in physically assembling the relevant molecular components, and have been exploited by evolution, pathogens, and cellular engineers to reshape cellular behavior. The $IP_{\beta II\text{-}C}$ s identified in this work may play a role in some of these cellular activities.

In summary, we have identified 17 human proteins or protein fragments that interact with βII-C, a region of the non-erythroid beta spectrin that is involved in spectrin tetramerization. Most of these proteins (14 of them) appear to interact with βII-C with lower affinity than that of αII-N since they do not interact with βII-C in the presence of αII-N. However, three of these proteins retain interactions with βII-C in the presence of αII-N, and one, the syntaxin binding protein fragment, abolishes αII-N and βII-C interactions. We suggest that further studies of these interactions, on structural and cellular levels, will provide a better understanding of brain physiology and pathophysiology.

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