Non-Erythroid Beta Spectrin: Effects of Mutations and of Interacting Proteins on Tetramerization

BY

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THESIS

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This thesis is dedicated to my parents,

Dr. Çiğdem Sevinç and Dr. Erdal Sevinç…

Without whom it would never have been accomplished…

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AS

TABLE OF CONTENTS

TABLE OF CONTENTS (*continued*)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

αI	erythroid alpha spectrin
αI-D1	first structural domain of erythroid alpha spectrin
αII	non-erythroid (brain) alpha spectrin
αII-D1	first structural domain of non-erythroid (brain) alpha spectrin
αII-N	a recombinant protein consisting of N-terminal 359 residues of αII
αII-N-V22Δ	a recombinant protein with a residue replacement at position 22 of αII-N
βI	erythroid beta spectrin
βI-C	a recombinant protein consisting of C-terminal residues (1898-2083) of βI
βII	non-erythroid beta spectrin
βII-C	a recombinant protein consisting of C-terminal residues (1697-2145) of βII
AD	activation domain of GAL4 transcriptional coactivator
BD	binding domain of GAL4 transcriptional coactivator
bp	base pairs
CD	circular dichroism
EPR	electron paramagnetic resonance
GST	glutathione-Sepharose 4B
Helix A[1]	the first helix of the first structural domain of αI-N1 or αII-N1
Helix A'	the first helix of the partial domain of βI-C1 or βII-C1

Helix B^1	the second helix of the first structural domain of αI-N1 or αII-N1
Helix B'	the second helix of the partial domain of βI-C1 or βII-C1
Helix C^1	the third helix of the first structural domain of αI-N1 or αII-N1
Helix A'	the lone helix of the partial domain of αI-N1 or αII-N1
IP	interacting protein
$IP_{\beta II\text{-}C}$	proteins interacting with βII-C
$IP_{\alpha II\text{-}N}$	proteins interacting with αII-N
IPTG	isopropyl-beta-D-thiogalactopyranoside
ITC	isothermal titration calorimetry
K_{av}	phase distribution coefficient
K_d	equilibrium dissociation constant
LB	lysogeny broth for *E. coli* growth
μM	micromolar
MCS1	first cloning site of the yeast three-hybrid plasmid, pBridge
MCS2	second cloning site of the yeast three-hybrid plasmid, pBridge
nM	nanomolar
pAD	yeast plasmid pGADT7
pBD	yeast plasmid pGBKT7
pBR	yeast three-hybrid cloning vector pBridge

PBS 7.4	5 mM phosphate buffer with 150 mM NaCl at pH 7.4
QDO	quadruple drop-out
SD	synthetic defined
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SPTAN1	gene coding for non-erythroid (brain) alpha spectrin
SPTBN1	gene coding for non-erythroid (brain) beta spectrin
TB	terrific broth for *E. coli* growth
TDO	triple drop-out
V_0	void volume of the mobile phase
V_E	elution volume
V_t	total liquid volume
WT	wild type
X-α-gal	5-bromo-4-chloro-3-indolyl-α-galactopyranoside
Y2H	yeast two-hybrid
YPDA	yeast growth medium with yeast extract, peptone, dextrose and adenine

SUMMARY

Spectrin performs its fundamental roles by forming a filamentous network beneath the plasma membrane, where the tetramerization of the spectrin heterodimers is crucial. We employed yeast two-hybrid (Y2H) methods to study the mutational effect of non-erythroid alpha spectrin (αII) at position 22 in tetramer formation with non-erythroid (beta) spectrin (βII), and to screen a human brain cDNA library to identify proteins interacting with βII-C, using a C-terminal fragment (residues 1697-2145) of non-erythroid beta spectrin (βII-C) as the bait. This region includes the tetramerization region involved in the association with alpha spectrin.

For the first part, interaction of wild type (αII-N) and 4 mutants (αII-N-V22D, -V22F, -V22M, and -V22W) of the first 359 residues at the N-terminal region with βII-C, were studied using colony growth and β-galactosidase activity assays of Y2H analysis simultaneously with isothermal titration calorimetry (ITC) analysis. Y2H results showed that the C-terminal region of βII interacts with the N-terminal region of αII, either the wild type, or those with V22F, V22M or V22W mutations. The V22D mutant did not interact with βII. For the positive results, we were not able to detect any differences in interactions between V22F, V22M or V22W with βII-C. Both colony growth rate and colony size, as well as the blue color indication for β-galactosidase activity did not show detectable differences between V22, V22F, V22M and V22W. ITC results showed that the K_d values for V22F were similar to those for the wild-type (about 7 nM), whereas the K_d values were about 35 nM for V22M and about 90 nM for V22W. We were not able to detect any binding for V22D with ITC methods. This study clearly demonstrates that the single mutation at position 22 of αII, a region critical to the function of non-erythroid α

spectrin, may lead to a reduced level of spectrin tetramers and abnormal spectrin-based membrane skeleton. These abnormalities could cause abnormal neural activities in cells.

For the second part, we identified 17 proteins that interacted with βII-C ($IP_{\beta II\text{-}C}$ s). The interacting 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) (Oh and Fung, 2007) 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 binding protein 1 fragment abolished αII-N and βII-C interaction. This suggests that this protein may inhibit or regulate non-erythroid spectrin tetramer formation.

CHAPTER 1

INTRODUCTION

A. Spectrin, A Multifunctional Protein

Spectrin is a prominent cellular protein that is ubiquitously expressed in vertebrates starting with simple metazoans, underlying its involvement in fundamental cellular processes (De Matteis and Morrow, 2000; Gascard and Mohandas, 2000; Kordeli, 2000; Bennett and Baines, 2001; Giorgi *et al.*, 2001; Djinovic-Carugo *et al.*, 2002). Originally identified from guinea pig erythrocytes forming a meshwork beneath the plasma membrane along with proteins such as actin, ankyrin, and protein 4.1 (Marchesi and Steers, 1968), spectrin was initially thought to be absent in other cell types (Hiller and Weber, 1977) until the identification of non-erythrocyte isoforms and their functions (Levine and Willard, 1981).

In addition to being a cytoskeletal protein, spectrin is involved in functions such as organization and maintenance of specialized plasma membrane domains at cell contacts and exocytosis (Bloch and Morrow, 1989; Lee *et al.*, 1993), protein sorting and accumulation (Pinder and Baines, 2000), neuritogenesis and neurotransmitter release (Beck, 2005), nuclear architecture during mitosis, organization of chromatin remodeling complexes, RNA processing, nucleoskeleton structure, or regulating DNase I function (McMahon *et al.*, 2001; Bettinger *et al.*, 2004), signal transduction (Gascard and Mohandas, 2000), DNA repair (Sridharan *et al.*, 2006), and active nuclear transport (Young *et al.*, 2003; Tang *et al.*, 2003).

B. Spectrin Isoforms

In human, two α- (I and II) and five β-spectrin (I - IV, and H) isoforms have been identified (Bennett and Baines, 2001) (Figure 1). The most common and well studied spectrin isoforms are erythroid αI- and βI-spectrins, and non-erythroid (brain) αII- and βII-spectrins (Bennett and Healy, 2008). Even though spectrin isoforms are expressed from different genes, all feature two common cardinal properties; they are made up of repeating spectrin structural domains, and they have a partial structural domain involved in tetramerization (Bennett, 2001).

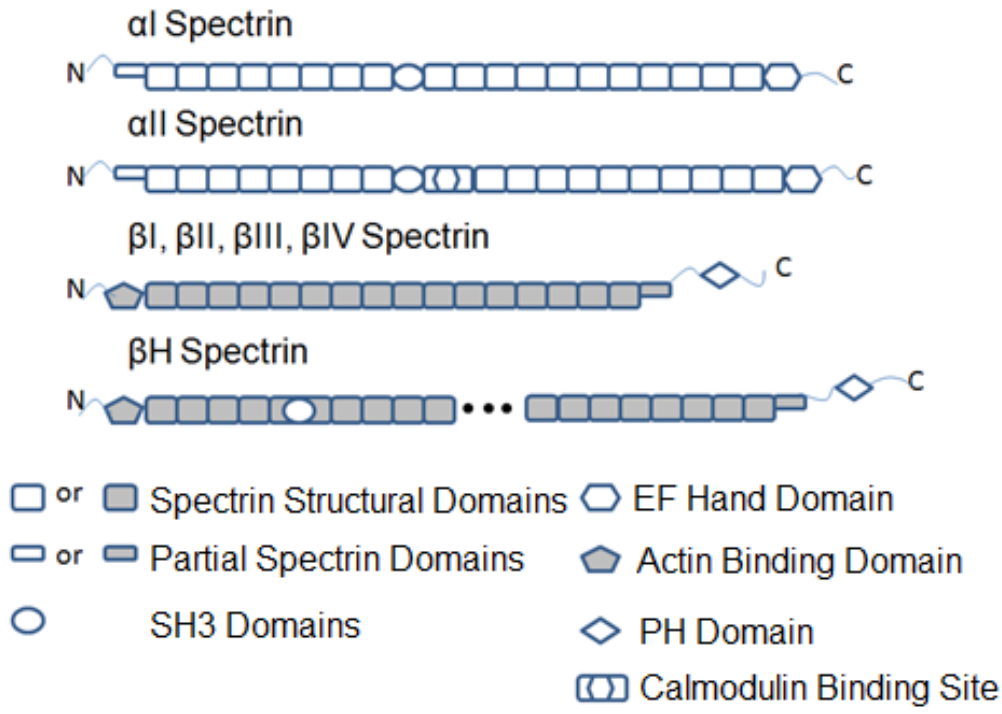

Figure 1. Schematic representation of the alpha and beta spectrin domain structures. Amino acid sequences outside the spectrin structural domains are designated with lines. This figure was prepared based on the descriptions in review article by Bennett and Baines, 2001.

Spatial localization of the spectrin isoforms in the cell is tightly regulated, for example, even though all four spectrin isoforms are expressed in neurons (Clark *et al.*, 1994; Winkelmann and Forget, 1993; Susuki and Rasband, 2008), αI- and βI-spectrin molecules are strictly localized in cell bodies, dendrites, postsynaptic terminals (Reiderer, 1988), and αII- and βII-spectrin in the axon and pre-synaptic termini (Kordeli, 2000). βIII spectrin was reported to be associated with golgi and cytoplasmic vesicles, and βIV, which is a newly discovered isoform, has been shown to localize at the axon initial segments and nodes of Ranvier of neurons (Stankiewich *et al.*, 1998; Berghs *et al.*, 2000; Tse *et al.*, 2001).

Each spectrin isoform, having molecular masses larger than 200 kDa, consists of tandemly homologous structural domains, which are triple helical bundled structures composed of approximately 106 amino acid residues (Djinovic-Carugo, 2002). Human α spectrin isoforms contain 22 such domains, and prior to the first domain is a single helix, Helix C', which is referred to as a partial domain. On the other hand β spectrin isoforms has 16 domains and a partial domain at the C-terminal end that has two helices, Helices B' and C'. Representative domain organization in spectrin isoforms is presented in Figure 1.

B.1. Alpha Spectrin

It has been shown that the C-terminal region of α spectrin associates with the N-terminal region of β spectrin to form a heterodimer, and two heterodimers in turn associate, at the N-terminal region of α spectrin on one heterodimer and C-terminal region of β spectrin on the other, to form tetramers (Cherry *et al.*, 1999; Begg *et al.*, 2000; Harper *et al.*, 2001; Speicher *et al.*, 2001). Even though spectrin isoforms exhibit high sequence similarity and identity (Mehboob *et al.*, 2003; Li and Fung, 2009; Mehboob *et al.*, 2010), the tetramerization affinity is lower in erythroid

isoforms, with the K_d in the μM range for model erythroid proteins, and relatively higher in non-erythroid (brain) proteins, with K_d in the nM range for model non-erythroid proteins (Bignone and Baines, 2003; Mehboob *et al*., 2003; Long *et al.*, 2007; Mehboob *et al.*, 2010).

Recently published crystal structure of the N-terminal region of αII spectrin, residues 1-147, a region containing the partial domain involved in the tetramerization of spectrin, Helix C', and the first structural domain (αII-D1), reveals several important features of non-erythroid brain spectrin (Mehboob *et al.*, 2010) (Figure 2).

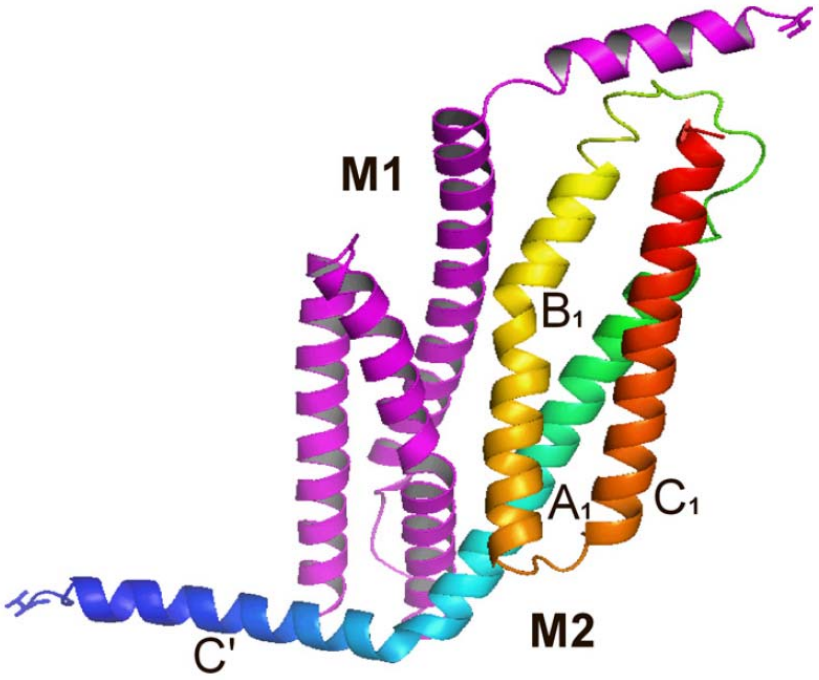

Figure 2. Crystal structure of the N-terminal 147 residues of non-erythroid (brain) spectrin at 2.3 Å resolution. Two monomers are observed in this asymmetric unit, where the ribbon structure of monomer 1 (M1) is shown in magenta, and of monomer 2 (M2) is shown in a spectrum of colors marking different regions of the protein (Mehboob *et al*., 2010).

The crystal structures show two slightly different conformations of the N-terminal 147 residues of αII spectrin. Each conformation contains a short unstructured segment followed by an unpaired helix, Helix C', with a bend, slight variations in its boundaries, and the location of the "bend" (Figure 2) (Mehboob *et al.*, 2010). Helix C', a region that is critical for the association with the C-terminal partial domain of β spectrin, starts with residue 12 or 14 and extends up to residue 36 in αII (Mehboob *et al.*, 2010), and starts with residue 21 and extends up to residue 45 in erythroid spectrin (αI) (Park *et al.*, 2003) (Table 1). Conformational mobility of Helix C' in αI spectrin, implies that this region may undergo conformational changes upon interacting with β spectrin (Antoniou *et al.*, 2008), as well as any other proteins that it may interact in the cells.

The junction region in both αI and αII, consists of 7 residues (46 - 52 in αI, and 37 - 43 in αII) that connects Helix C' to the first structural domain (αI-D1 or αII-D1) (Mehboob *et al.*, 2010). This region was shown to be in a helical conformation in αII, but in an unstructured conformation in αI which undergoes a conformational change (to a helical conformation) upon binding to βI spectrin (Mehboob *et al.*, 2003; Park *et al.*, 2003; Li and Fung, 2009; Ipsaro *et al.*, 2010; Mehboob *et al.*, 2010). We believe, this difference modulates the association affinity of these isoforms with β-spectrin.

The first structural domain of both αI and αII spectrin is composed of a triple helical bundle, where three helices (Helix A_1, Helix B_1, and Helix C_1) form the first triple helical domain of α spectrin (αI-D1 and αII-D1). Helix A_1 contains residues 53 - 81 in αI-D1, and residues 44 - 66 in αII-D1. The loop region connecting the first helix (Helix A_1) to the second helix (Helix B_1) in the first structural domain spans residues 82 - 87 in αI, and 67 - 77 in αII. Helix B_1 contains residues 88 - 118, and residues 78 - 111 or 112 in αII-D1. The loop region connecting Helix B_1

to Helix C_1 spans residues 119 - 122 in αI and 112 or 113 - 116 in αII. And finally, Helix C_1 contains residues 123 - 153, and residues 117 - 143 or 146 in αII-D1 (Table 1). Despite the similarities of the boundaries in other domains, it is interesting to note that the region connecting Helices A_1 and B_1 is relatively long in αII spectrin, 11 residues, whereas it is only 6 residues in αI spectrin. Also the loop connecting Helix B_1 and C_1 is only 4 or 5 residues, which is still much shorter . Therefore, it is logical to speculate that this long loop in αII may provide binding sites for other nonspectrin proteins to recognize this specific site for interaction. This region consists of mainly polar residues, with differing side chain properties, which is well suited for specific molecular recognition and interactions. The first few amino acids that have not been mentioned in Table 1 were either not studied or not seen (Mehboob *et al.*, 2010).

TABLE I. Secondary structure summary of αI, αII, βI, and βII spectrin isoforms (Park *et al.*, 2003; Mehboob *et al.*, 2010; Song *et al.*, 2011).

	αI (1 - 156)	**αII (1 - 147)**	**βI (1898 - 2083)**	**βII (2016 - 2091)**
Helix C_1	123 - 153	117 - 143/146		
Loop B_1C_1	119 - 122	112/113 - 116		
Helix B_1	88 - 118	78 - 111/112		
Loop A_1B_1	82 - 87	67 - 77		
Helix A_1	53 - 81	44 - 66		
Junction Region	46 - 52	37 - 44		
Helix C'	21 - 45	12/14 - 36		
Unstructured	14 - 20	8/11 - 11/13	2071 - 2083	
Helix B'			2042 - 2070	2047 - 2091
Loop B'A'			2034 - 2041	2042 - 2048
Helix A'			2083 - 2033	2016 - 2041

The atomic resolution structure of αII-D1 shows multiple hydrogen bonds spread through the interfaces of the three helices stabilizing the triple coiled coil structure, in addition to the hydrophobic clusters in the structure (Song *et al.*, 2009; Mehboob *et al.*, 2010). However, a higher number of specific hydrogen bonds were identified in αII-D1, compared to αI-D1, which may contribute to the higher thermal stability of the αII-D1 compared to αI-D1 (Mehboob *et al.*, 2010).

Two hydrophobic clusters have been identified in the Helices A'-B'-C' complex of αII / βII. The first hydrophobic cluster involves three residues of Helix C' (Ile-15, Val-22, and Leu-23). The second hydrophobic cluster involves L40 of Helix C' and V2052, L2055, and I2056 of βII (Mehboob *et al.*, 2010).

It is important to note that residue Val-22 in Helix C' of αII, is homologous to a clinical hotspot in αI (V30) (Lecomte *et al.*, 1993) (Details in Chapter 2). Residue 22 in αII corresponds to a "*d*" position in the heptad repeat and is a member of the hydrophobic cluster in the interface of the triple helical bundle (Mehboob *et al.*, 2010). As presented in Chapter 2, we replaced the hydrophobic valine residue, with other amino acid residues that exhibit different side chain properties, and observed the effect of this replacement on the tetramerization affinity of spectrin. These mutants were V22W, V22M, V22D, V22F.

In separate studies, we have used ITC methods to determine K_d values of α/β heterodimer association to form tetramers in model systems (*e.g.,* Mehboob *et al.*, 2010; Kang *et al.*, 2010; Lam *et al.*, 2009; Li an Fung, 2009; Mehboob *et al.*, 2003). Previously, our yeast two-hybrid studies showed that the mutation of αII at position Arg-37 to a proline residue impairs its ability to form tetramers with the C terminal domain of βII (Sumandea ad Fung,

2005). Consequent studies showed that mutation of αII at position Arg-37 increases the K_d value from about 9 nM for αII with beta I spectrin (βI) to 10 μM for the R37P mutation (Mehboob *et al.*, 2005). The participation of this residue in the hydrophobic cluster may explain the impact of this replacement on the affinity, in addition to the possible contribution of the low helical propensity of the proline amino acid residue.

Accumulated molecular knowledge on the spectrin tetramerization shows that it is important to underline that, ITC analysis of the titration of αI-N1 with βI-C1 or βII-C1 showed similar titration isotherms with a K_D value of ~1 μM (Mehboob *et al.*, 2003; Long *et al.*, 2007; Lam *et al.*, 2009; Song *et al.*, 2011), and similarly, the binding of αII-N1 with βI-C1, or βII-C1 showed similar binding isotherms with a K_d value of ~10 nM (Long *et al.*, 2007; Li and Fung, 2009; Song *et al.*, 2011). These results indicate that β-spectrin I and II does not contribute to the observed differences in dimer association to form tetramers; the differences are due to the α-spectrin component of the interaction (Mehboob *et al.*, 2010; Song *et al.*, 2011). Therefore, βI- and βII-spectrin not only exhibit 80% sequence similarity but similar affinities for α-spectrin isoforms for forming tetramers.

B.2. Beta Spectrin

As indicated in an earlier publication (Song *et al.*, 2011), majority of the structural information available for β spectrin is for either for structural domains (e.g., Yan *et al.*, 1993; Pascual *et al.*, 1997; Davis *et al.*, 2009; Ipsaro *et al.*, 2010) or SH3 domains (e.g., Chevelkov *et al.*, 2005; Prokhorov *et al.*, 2008; Gushchina *et al.*, 2009). For the tetramerization regions, most of the studies have focused on the N-terminal region of αI- and αII-spectrin (Mehboob *et al.,* 2003; Park *et al.*, 2003; Chevelkov *et al.*, 2005; Long *et al.*, 2007; Antoniou *et al.*, 2008; Lam *et al.*, 2009; Li and Fung, 2009; Mehboob *et al.*, 2010), but only few have studied the C-terminal region of β-spectrin (Nicolas *et al.*, 1998; Luo *et al.*, 2001; Mehboob *et al.*, 2005; Bignone *et al.*, 2007; Ipsaro *et al.*, 2010; Song *et al.*, 2011).

The high resolution structures of the C-terminal tetramerization region of free βI- or βII-spectrin proteins are not known. Spin label electron paramagnetic resonance (EPR) studies has shown that the Helix B' in the partial domain of βI spectrin ends at residue 2070 (Luo *et al.*, 2001). The crystal structure of the C-terminal region of erythroid β-spectrin bound to αI has been solved by X-ray diffraction (Ipsaro *et al.*, 2010) (Table 1).

In a recent study, homology structures of the C-terminal fragments of βI and βII spectrin (residues 1898 – 2083 and residues 1906 – 2091, respectively) were modeled using a recombinant protein of domains 16 – 17 of chicken α-spectrin (PDB code: 1CUN; Grum, 1999) as the template (Song *et al.*, 2011) (Figure 3). Both structures featured a triple helical bundle as the full structural domain, which was followed by the partial domain, Helices A' and B' (Figure 3). An important structural feature of βI spectrin was that, the last helix in the structural domain (Helix C_{16}) and first helix in the partial domain (Helix A') showed no clear boundary between

them, with one helix merging into the next (Song *et al.*, 2011). Similar overall structures were obtained except for Helix B', which was 16 residues longer in βII (residues 2042-2070 in βI and residues 2047-2091 in βII). This region in βI was followed by an unstructured region, which was helical in βII. It is believed that these structural differences in β-spectrin C-terminal region lead to specific binding of proteins to βI and not to βII, and similarly to βII and not to βI (Song *et al.*, 2011).

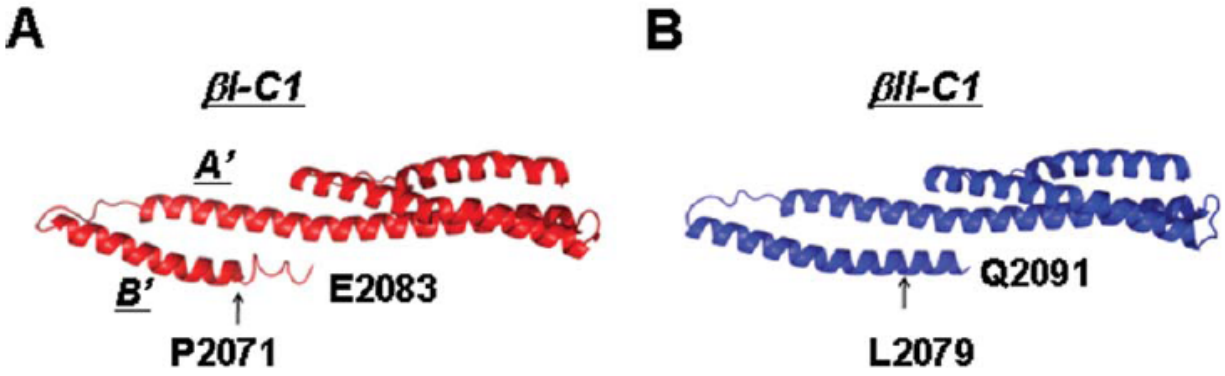

Figure 3. Predicted three-dimensional structures of β-spectrin segments. The structures of the C-terminal fragment (residues 1898 – 2083) of βI-C1 (A) and (residues 1906 – 2091) of βII-C1 (B). Both structures feature a canonical triple helical bundle that is characteristic for the spectrin repeats, and the double helical partial domain. The major difference is following residue P2071 of βI-C1, which assumes unstructured conformation after this residue, whereas βII continues with a helical conformation (Song *et al.*, 2011).

The predicted structure of the Helix B' in free βI-C1 (not associated with αI spectrin) shows a good overlap with previously obtained information on a local region in its structure using spin label EPR studies (Luo *et al.*, 2001; Mehboob *et al.*, 2005; Mehboob *et al.*, 2011). Both studies show that the residues before P2071 are in helical conformation, whereas the residues after P2071 are in an unstructured conformation. On the other hand, a recent study showed that the large portion of the unstructured region (residues 2071 - 2083) in the free form is helical (2074 - 2083) in the bound form. Structural differences in the free (Park *et al.*, 2003) and bound (Long *et al.*, 2007; Antoniou *et al.*, 2008; Song *et al.*, 2009) forms have been observed experimentally. This is coinciding with the conformational change in the N-terminal junction region of αI-spectrin that changes into a helical conformation in the bound state from an unstructured conformation in the unbound state (Antoniou *et al.*, 2008; Ipsaro *et al*, 2010).

Based on the differences in hydrogen bond networks and/or pairs of cation-Π interactions between the side chains of a particular β-spectrin isoform and its interactor (Song *et al.*, 2011), it is possible that a specific cellular protein may bind to one β-spectrin isoform, maintain or regulating its tetramerization with a specific α spectrin isoform. In addition to regulating the tetramerization, such interactions may regulate the localization of the β spectrin isoforms, for example in hair cells (Legendre *et al.*, 2008) and in neuronal cells (Bignone *et al.*, 2007).

Structural differences between different βI and βII spectrin isoforms may very well extend beyond the regions discussed above (Song *et al.*, 2011). For example, two important residues in βII spectrin, residue 2110 (serine) and 2159 (threonine) can be phosphorylated which leads to a great reduction in its affinity for α spectrin isoforms. However, the first residue is not conserved in βI spectrin, which may point to different regulation mechanisms, or interactions of different

spectrin isoforms (Bignone *et al.*, 2007). All these differences discussed above may provide basis for the selective interaction of β spectrin isoforms with different α spectrin isoforms, as well as with other non-spectrin proteins.

Three proteins have been identified to bind βI and βII spectrin in a phage displayed screening (Song *et al.*, 2011). Two of them selectively binds to βI-C1, and one binds to βII-C1. Modeling studies show the interaction site for the two proteins at the unstructured region of βI-C1. Along with other structural comparison, results obtained from that study points to the similarity of βI and βII spectrins at their tetramerization sites, however differences at the downstream regions, which would enable the selective binding of the proteins that they identified in their phage displayed screening study, as well as for other proteins.

Spectrin isoforms interact with numerous cellular proteins (nonspectrin proteins) in order to perform some of the cellular functions discussed above. Several proteins interacting with the N-terminal region (residues 1 - 359) of αII spectrin (αII-N) have been identified in a library screening in our laboratory (Oh and Fung, 2007). These proteins were Duo protein, Lysyl-tRNA-synthetase, TBP-associated factor 1, two isoforms (b and c) of protein kinase A interacting protein and two different segments of Zinc finger protein 333. The interaction of three proteins were shown to be abolished by a mutation on αII-N, which also abolishes spectrin tetramerization (Sumandea and Fung, 2005). We also found out that these proteins may compete with its spectrin partner to regulate spectrin tetramerization and cytoskeletal structures.

Several other proteins have been identified to interact with αII spectrin. A partial view of αII (SPTAN1) putative interactome from NCBI protein interaction databases is schematically presented in Figure 4, using STITCH 2.0 software (to populate the list of interactions GENE;

Genes and mapped phenotypes service of NCBI (www.ncbi.nlm.nih.gov/gene) was used with an "SPTAN1" inquiry. STITCH 2.0 (Search Tool for Interactions of Chemicals) was used at; www.stitch.embl.de to visualize the interactions).

Close examination of the cellular functions of the proteins listed above, indicates that these proteins exhibit multiple types of functions. These interactions underline the involvement of spectrin isoforms in diverse cellular processes.

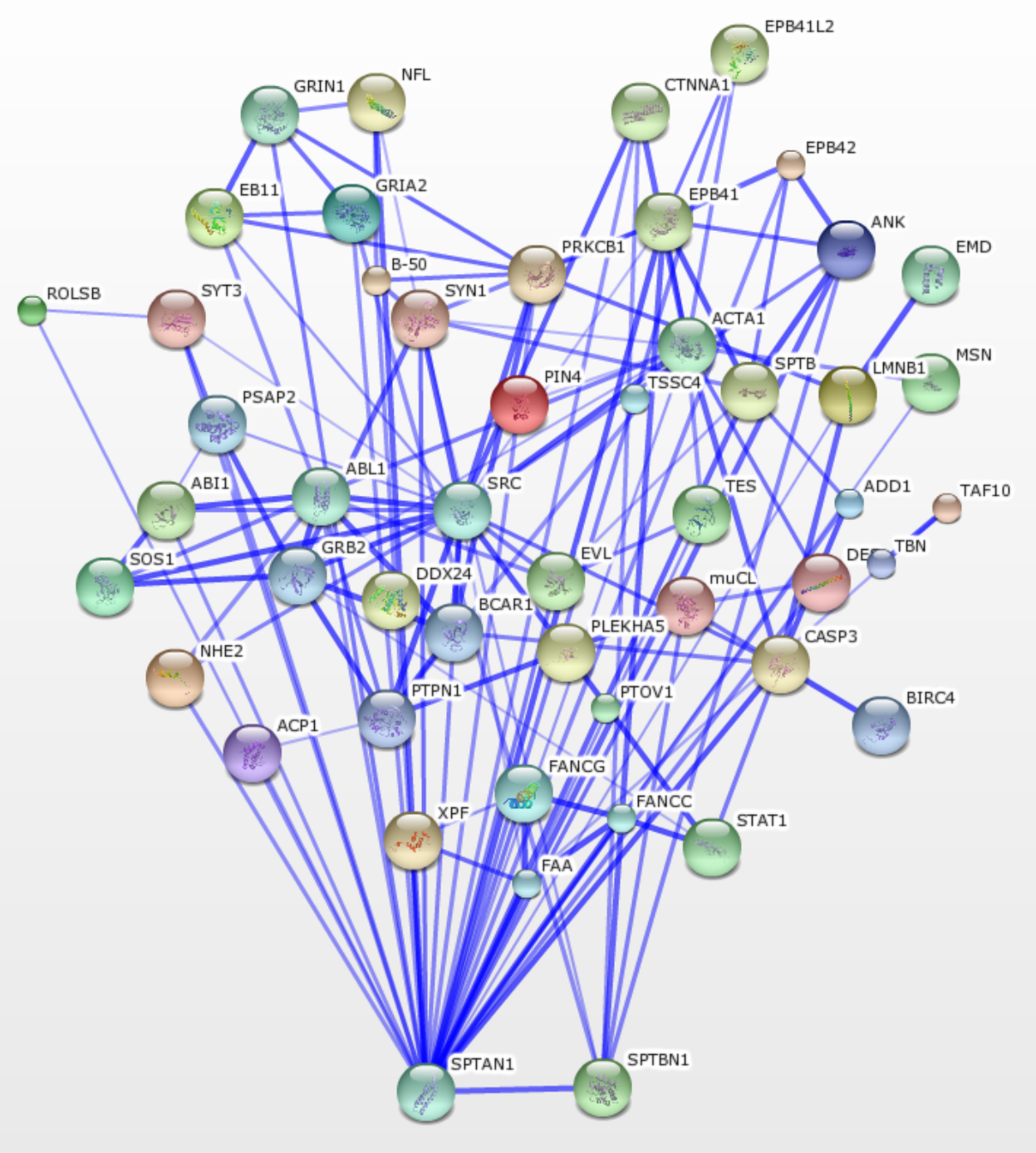
EPB41L2
NFL
GRIN1
CTNNA1
EPB42
EB11
GRIA2
EPB41
ANK
PRKCB1
EMD
B-50
ROLSB
SYT3
SYN1
ACTA1
SPTB
LMNB1
MSN
PIN4
TSSC4
PSAP2
ABL1
ABI1
SRC
TES
ADD1
TAF10
GRB2
SOS1
EVL
TBN
DDX24
muCL
BCAR1
PLEKHA5
CASP3
NHE2
PTPN1
PTOV1
BIRC4
ACP1
FANCG
FANCC
XPF
STAT1
FAA
SPTAN1
SPTBN1

Figure 4. Proteins interacting with brain alpha spectrin (SPTAN1) from NCBI protein interaction databases. The details and the references of these proteins are given in the web-site, but not listed here. The proteins are; abl-interactor 1 (ABI1), acid phosphatase 1 (ACP1), actin, both alpha 1 and beta subunit (ACTA1), Adducin 1 (ADD1), ankyrin 1 (ANK1), calpain 1, large subunit (CAPN1), c-abl oncogene 1, non-receptor tyrosine kinase (ABL1), apoptosis related cysteine kinase peptidase (caspase3) (CASP3), alpha 1 subunit of catenin (CTNNA1), DEAD box polypeptide 24 (DDX24), desmin (DES), disrupted in schizophrenia 1 (DISC1), emerin (EMD), Enah/Vasp-Like (EVL) protein, testis derived transcript 3 (TES), erythrocyte membrane protein 4.1 (EPB41), erythrocyte membrane protein 4.1-like 2 (EPB41L2), erythrocyte membrane protein band 4.2 (EPB42), excision repair cross-complementing rodent repair deficiency, complementation group 4 (ERCC4), Fanconi anemia complementation group A (FANCA), Fanconi anemia complementation group C (FANCC), Fanconi anemia complementation group G (FANCG), growth associated protein (GAP43), glutamate receptor, ionotropic (AMPA2) (GRIA2), glutamate receptor, ionotropic, N-methyl D-aspartate 1 (GRIN1), glutamate receptor, ionotropic, N-methyl D-aspartate 2D (GRIN2D), lamin B1 (LMNB1), moesin (MSN), neurofilament, light polypeptide (NEFL), peptidylprolyl cis/trans isomerase - NIMA-interacting 4 (PIN4), phosphodiesterase 4D (PDE4D), plectin (PLEC), pleckstrin homology domain containing, family A member 5 (PLAKHA5), protein kinase C, beta (PRKCB), prostate tumor overexpressed 1 (PTOV1), SH3 and multiple ankyrin repeat domains 1 (SHANK1), SH3 and multiple ankyrin repeat domains 3 (SHANK3), solute carrier family 9, member 2 (SLC9A2), son of sevenless homolog 1 (SOS1), erythroid spectrin beta subunit (SPTB), non-erythroid spectrin beta subunit (SPTBN1), v-src sarcoma (Schmidt-Ruppin A-2)

viral oncogene homolog (SRC), signal transducer and activator of transcription 1 (STAT1), synapsin 1 (SYN1), TATA box binding protein-associated factor (TAF), tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1 (TANC1), tumor suppressing subtransferable candidate 4 (TSSC4), vimentin (VIM).

Similarly, numerous interacting partners of βII spectrin, mostly related to its important cellular functions, have been identified. For example, using yeast two-hybrid systems, neurofibromatosis 2 tumor suppressor schwannomin was shown to interact with βII, which is regulated by alternative splicing of the schwannomin protein (Scoles et al., 2001).

A partial view of βII (SPTBN1) putative interactome from NCBI protein interaction databases is schematically presented in Figure 5, again, using STITCH 2.0 software to represent interactions deposited in GENE (Genes and mapped phenotypes) service of NCBI (www.ncbi.nlm.nih.gov/gene). αII protein, and its interactions with βII interacting proteins is represented to show common interactions.

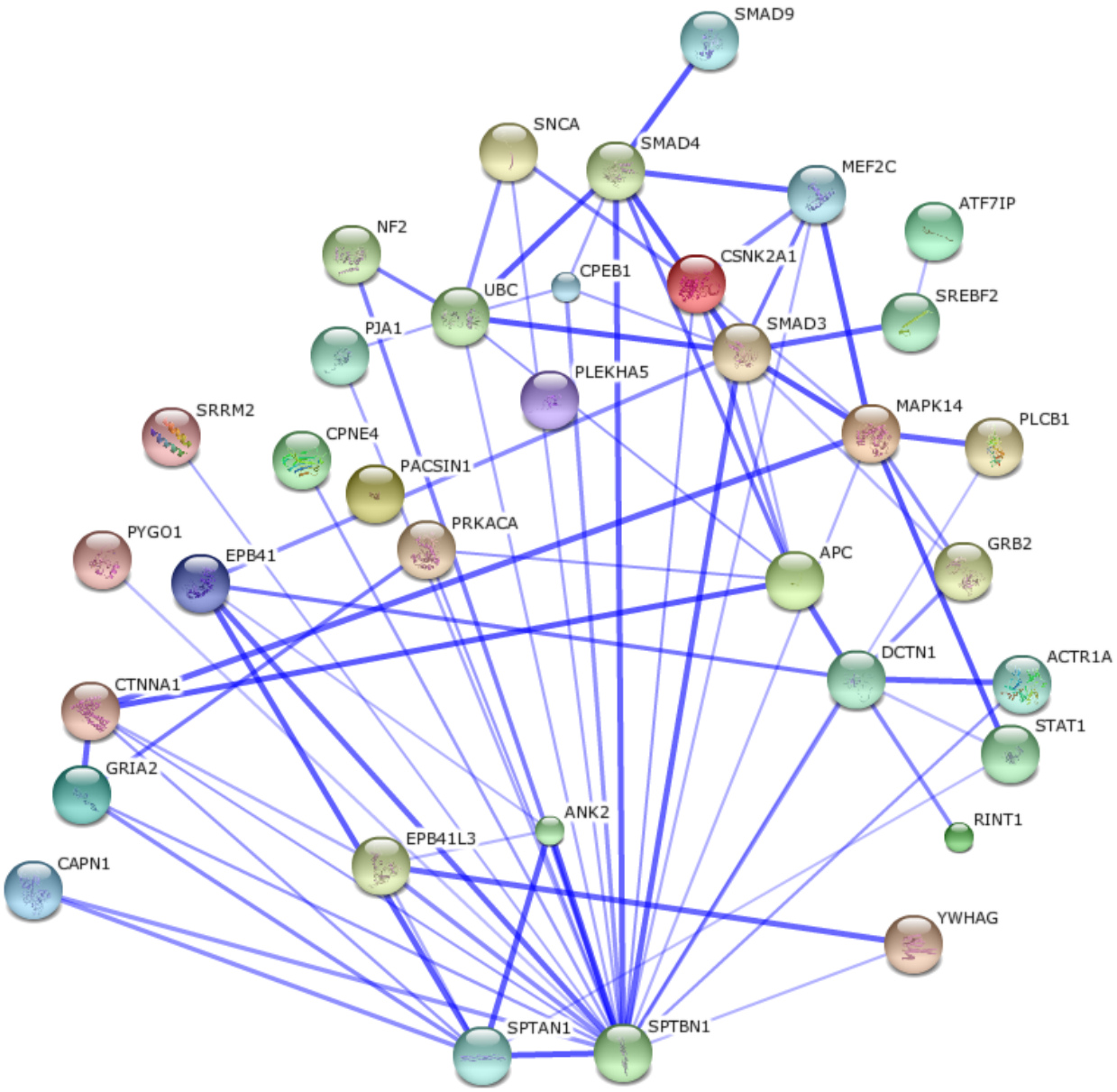
SMAD9
SNCA
SMAD4
MEF2C
ATF7IP
NF2
CSNK2A1
CPEB1
UBC
SREBF2
PJA1
SMAD3
PLEKHA5
SRRM2
MAPK14
PLCB1
CPNE4
PACSIN1
PYGO1
PRKACA
EPB41
APC
GRB2
DCTN1
ACTR1A
CTNNA1
STAT1
GRIA2
ANK2
RINT1
EPB41L3
CAPN1
YWHAG
SPTAN1
SPTBN1

Figure 5. Proteins interacting with brain beta spectrin (SPTBN1) from NCBI protein interaction databases. The details and the references of these proteins are provided in the web-site, but not listed here. The proteins are; YWHAG, 14-3-3 gamma; ATF7IP, activating transcription factor 7 interacting protein; APC, Adenomatus polyposis coli (APC) protein; alpha-Cat, Alpha-catenin; ANK2, Ankyrin-B; CAPN1, Calpain; CSNK2A1, Casein kinase 2; C/EBP, CCAAT/enhancer binding protein; ACTR1B, Centractin; CPNE (1&4), Copine family of proteins (isoforms 1 and 4); GRID, Delta glutamate receptor; MEF2C, Myocyte enhancer factor 2C; NF2, Neurofibromin 2; PLCB1, Phospholipase C, beta 1; PLEKHA5, Pleckstrin homology domain containing, family A member 5; PJA1, Praja ring finger 1; EPB41L3, Protein 4.1R; PKC, Protein kinase C; PACKSIN1, Protein kinase C/casein kinase substrate in neurons; PYGO1, Pygopus homolog 1; RINT1, RAD50 interactor 1 protein; SRRM2, Serine/arginine repetitive matrix 2; STAT1, Signal transducer and activator of transcription 1; SMAD3, 4, and 9, Mothers against decapentaplegic homolog isoforms 3, 4, and 9; SREBF2, Stereol regulatory element binding transcription factor 2; SYNCA, Synuclein, alpha; UBC, Ubiquitin c.

It is interesting to note that the number of proteins identified to interact with αII spectrin is much higher than the number of proteins interacting with βII spectrin.

Proteins interacting with spectrin isoforms may regulate cellular functions of spectrin. However, in addition to these interactions, others may be responsible for the regulation of the spectrin tetramerization. It is interesting to note that, several recent studies mapped hereditary anemia mutations to the interacting domains in erythroid spectrin (Lam *et al.*, 2009; Song *et al.*, 2009; Ipsaro *et al.*, 2010). Structural information of αII spectrin show that the mutations in αII spectrin may alter the equilibrium between the spectrin dimers ad tetramers in the cell, leading to reduced levels of functional tetramers and increased levels in dimers, which is regarded as a potential cause for abnormal neuronal functions (Mehboob *et al.*, 2010). Therefore, further understanding of the regulation of the spectrin tetramerization is crucial.

C. Spectrin Tetramers

The functional form of spectrin in the cytoskeleton is a tetramer (Reiderer *et al.*, 1988), and tetramerization involves helical bundling of the single helix (Helix C') at the N-terminal region of an α spectrin in one αβ heterodimer, and two helices (Helix A' and Helix B') at the C terminal region of β spectrin from another αβ heterodimer. This interaction recapitulate formation of a composite 3-helix bundle structural domain as shown in erythrocyte spectrin (Speicher *et al.*, 1980; Park *et al.*, 2003; Mehboob *et al.*, 2003; Long *et al.*, 2007; Antoniou *et al.*, 2008; Song *et al.*, 2009; Mehboob *et al.*, 2010; Ipsaro *et al.*, 2010) (Figure 6).

Detailed structural information is required for a complete understanding of the tetramerization of spectrin isoforms.

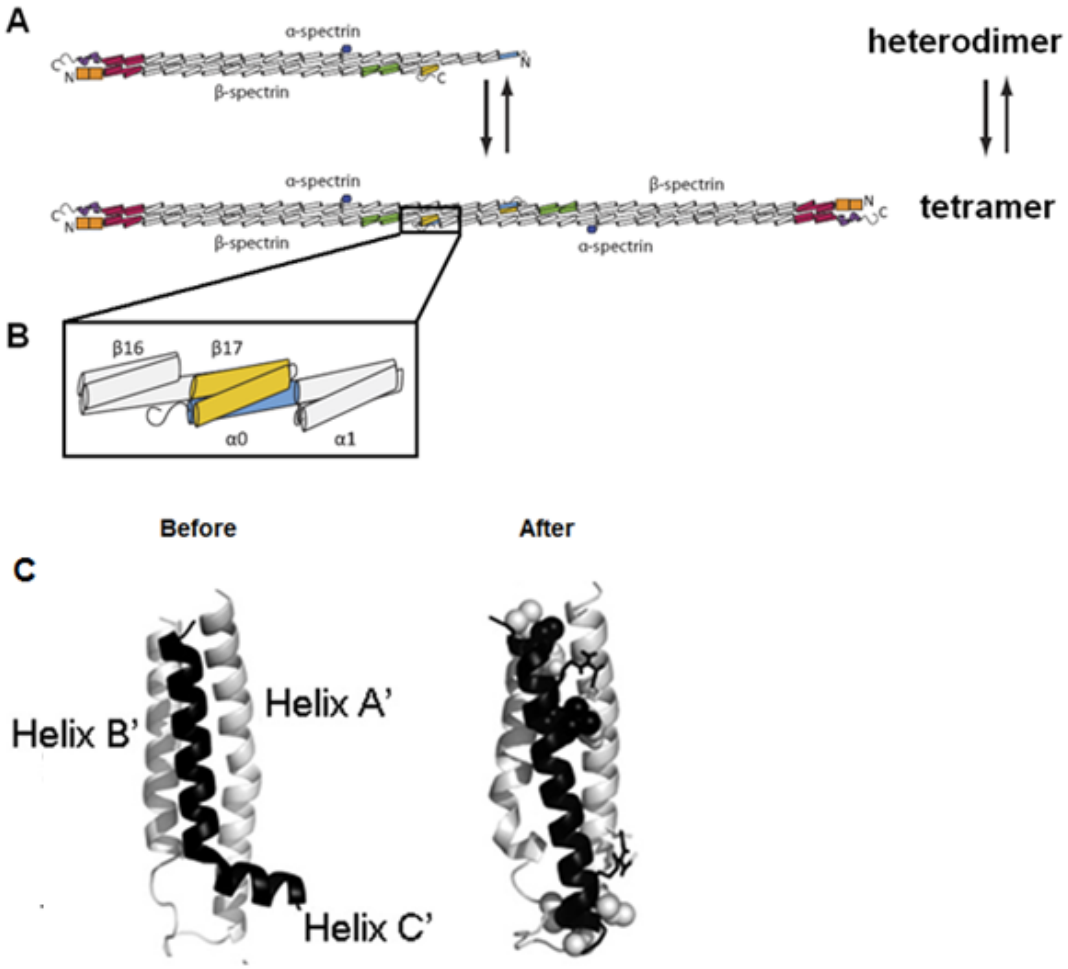

Figure 6. Schematic representation of spectrin tetramerization. A. Spectrin assembly starts with the formation of the spectrin heterodimers and continues with head-to-head interaction of two heterodimers to form spectrin tetramers via interaction at the N-terminal region of α spectrin, and C-terminal region of β spectrin (region marked with a box). B. An enlarged schematic representation of the molecular components of tetramerization. C. Homology model of αII/βII tetramerization region created using a published structure of a structural domain, subjected to molecular dynamics simulations (labeled *before* and *after*) (modified from Ipsaro *et al.*, 2010; Mehboob *et al.*, 2010).

Previously, we identified several mutations at the tetramerization region of non-erythroid (brain) alpha spectrin (αII) that impair its interaction with βII spectrin (Sumandea and Fung, 2005). Similar mutations in erythroid spectrin (αI) leading to reduced amounts of tetramers leading to hereditary elliptocytosis (Tse and Lux, 1999).

Given the importance of tetramerization of spectrin isoforms, the regulation of spectrin tetramerization emerges as a possible venue for the regulation of several cellular roles of spectrin. For example, the tetramerization of spectrin isoforms was shown to be essential for neuritogenesis (Bignone *et al.*, 2007). Polarized spectrin assembly via spatial regulation of the tetramerization in epithelial cells shed new light on of cell polarity and the contributions of spectrin (Lee *et al.*, 1997). In myocardial cells, αII spectrin distribution is coincident with Z-discs and plasma membrane of myofibrils (Bennett *et al.*, 2004). Identification of proteins that regulate spectrin tetramerization through interactions with spectrin isoforms is of utmost importance for understanding the physiology and the pathophysiology of human brain.

In this study, we first aimed to understand the mutational effects on spectrin tetramerization. For this we used both the yeast two-hybrid (Y2H) system and ITC methods to further study the effect of mutations at position 22 of αII tetramer formation.

Secondly, we aimed to identify proteins interacting with a C-terminal fragment (residues 1697 – 2145) of βII-spectrin (βII-C), which includes tetramerization region. We also studied the effects of these proteins on αII-N and βII-C association to identify proteins that may regulate non-erythroid spectrin tetramerization.

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CHAPTER 2

YEAST TWO-HYBRID AND ITC STUDIES OF ALPHA AND BETA SPECTRIN INTERACTION AT THE TETRAMERIZATION SITE

This chapter was submitted to "Cellular and Molecular Biology Letters" journal (Sevinc, A., Witek, M. A., and Fung, L. W.-M.). It has been accepted with minor revisions

A. Abstract

Yeast two-hybrid (Y2H) and isothermal titration calorimetry (ITC) methods were used to further study the mutational effect of non-erythroid alpha spectrin (αII) at position 22 in tetramer formation with beta spectrin (βII). Four mutants, αII-V22D, V22F, V22M and V22W, were studied. For the Y2H system, we used plasmids pGBKT7, consisting of the cDNA of the first 359 residues at the N-terminal region of αII, and pGADT7, consisting of the cDNA of residues 1697 - 2145 at the C-terminal region of βII. Strain AH109 yeast cells were used for colony growth assays and strain Y187 was used for β-galactosidase activity assays. Y2H results showed that the C-terminal region of βII interacts with the N-terminal region of αII, either the wild type, or those with V22F, V22M or V22W mutations. The V22D mutant did not interact with βII.

For ITC studies, we used recombinant proteins of the αII N-terminal fragment and of the erythroid beta spectrin (βI) C-terminal fragment; results showed that the K_d values for V22F were similar to those for the wild-type (about 7 nM), whereas the K_d values were

about 35 nM for V22M and about 90 nM for V22W. We were not able to detect any binding for V22D with ITC methods. This study clearly demonstrates that the single mutation at position 22 of αII, a region critical to the function of non-erythroid α spectrin, may lead to a reduced level of spectrin tetramers and abnormal spectrin-based membrane skeleton. These abnormalities could cause abnormal neural activities in cells.

B. Introduction

Spectrin, a prominent cytoskeletal protein, exerts its fundamental role in cells by forming a sub-membrane filamentous network. An essential aspect of the spectrin network formation is the tetramerization of spectrin αβ heterodimers. We have previously used the yeast two-hybrid system and random mutagenesis to investigate the effects of amino acid mutations on the tetramerization of non-erythroid (brain) spectrin (fodrin) (Sumandea and Fung, 2005). The Y2H techniques have been developed as convenient and useful methods to screen for protein interactors (Fields and Song, 1989; Fields, 2009; Hu *et al.*, 2009), particularly when libraries of vectors containing protein cDNAs are commercially available. We have used such methods to identify some interactors of non-erythroid alpha spectrin (αII) (Oh and Fung, 2007). These studies are often qualitative in nature - a protein either interacts or does not interact with another protein. However, several studies report quantitative results from Y2H studies. For example, colonies of Y2H system with common polymorphisms of *BRCA1* from cancer predisposing mutations were considerably smaller than controls (Humphrey *et al.*, 1997), colony growth rates (cell viability) correlate with the strengths of interactions (Estojak *et al.*, 1995; Jabbour *et al.*, 2006), the levels of

transcription activation correlate with the strength of the binding interaction in a "small colony phenotype", a growth phenotype discovered serendipitously (Coyne *et al.*,2004), and β-galactosidase activities correlate with protein-protein interaction affinities (Stavolone *et al.*, 2001; Ma *et al.*, 2003). Yet, some authors indicate that, "our results emphasize the difficulty of attempting to quantitate differences in affinity from two-hybrid experiments alone" (Estojak *et al.*, 1995). Others show that Y2H results do not correlate with protein affinities (Larin *et al.*, 1999; Grootjans *et al.*, 2000; Crowthler *et al.*, 2005). Since protein expression, structures and nature of interaction may vary from system to system in Y2H systems, many studies have focused on studying single mutation effects on protein-protein interactions (*e.g.*, Coyne *et al.*, 2004).

In our study, we used both the Y2H system and ITC methods to further study the mutational effect of αII at position 22 on tetramer formation. Previously we have used ITC methods to determine K_d values of α/β heterodimer association to form tetramers in model systems (*e.g.,* Mehboob *et al.*, 2010; Kang *et al.*, 2010; Lam *et al.*, 2009; Li an Fung, 2009; Mehboob *et al.*, 2003). Recently, we found that mutation of αII at position 37 increases the K_d value from about 9 nM for αII with beta I spectrin (βI) to 10 μM for the R37P mutation (Mehboob *et al.*, 2005). Residue 22 in αII corresponds to a "*d*" position in the heptad repeat and is in the interface of the triple helical bundle in α/β tetramers Mehboob *et al.*, 2010). In this study, we found that the mutation effect was most severe for V22D, followed by V22W and V22M, whereas little effect was observed for V22F.

C. Methods

C.1. Yeast Two-Hybrid Assays

The Y2H system with colony growth and β-galactosidase detection methods were used to determine the interaction between βII and αII, wild-type or its mutants, at the tetramerization region. The Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA) was used. The yeast strain Y187, which is auxotrophic for leucine and tryptophan with Gal4-inducible *lacZ* gene, or strain AH109, which is auxotrophic for adenine, histidine, leucine, lysine, tryptophan and uracil and with Gal4-inducible *lacZ* genes was used. Plasmids pGBKT7 (pBD) with the cDNA of the non-erythroid alpha spectrin (αII) consisting of the first 359 residues at the N-terminal region (αII-N) (pBD-αII-N) and pGADT7 (pAD) with the cDNA of beta-spectrin consisting of residues 1697-2145 at the C-terminal region (βII-C) (pAD-βII-C) were previously prepared (Sumandea and Fung, 2005). Plasmids of two mutations at position 22 of αII-N, V22W and V22M, prepared by standard methods (Mehboob *et al.*, 2005) as well as two previously prepared mutants (V22D and V22F) (Sumandea and Fung, 2005) (pBD-αII-N-V22Δ) were also used.

For the colony growth assay, AH109 cells with pAD-βII-C and pBD-αII-N, or pBD-αII-N-V22Δ, were grown at 30 °C on agar plates with a growth medium containing all essential amino acids but tryptophan, leucine and histidine, and lacking adenine (SD/-W/-L/-H/-A with SD Minimal Agar Base and -Leu/-Trp/-His/-adenine DO Supplement, both from Clontech) for three days before photography. Under this high-stringency growth condition, cells with strongly interacting protein pairs grow and form colonies, whereas colonies with proteins with low-affinity interactions may be missed (Clontech user manual). We also

prepared pAD-βI-C, with βI-C consisting of residues 1898 - 2083 of βI and performed colony growth assay with pBD-αII-N or pBD-αII-N-V22D.

For β-Galactosidase assay via colony lift method, strain Y187 cells with pAD-βII-C and pBD-αII-N, or pBD-αII-N-V22Δ, were grown at 30 °C on agar plates with a growth medium containing all essential amino acids but leucine and tryptophan (SD/-Leu/-Trp with SD Minimal Agar Base and -Leu/-Trp DO Supplement; both from Clontech) for three days before colony lifting steps, as described in the manufacturer user manual. Cells with interacting protein pairs produce β-galactosidase to give a blue color on filter papers when soaked with a solution consisting of its substrate, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal soaking solution, see Clontech user manual).

C.2. Isothermal Titration Calorimetry

Recombinant proteins αII-N, αII-N-V22Δ (V22D, V22F, V22M and V22W) and βI-C were prepared, following standard laboratory techniques (Mehboob *et al.*, 2010). Briefly, protein expression vector pGEX-2T was used to express glutathione S-transferase fusion protein, and purified with affinity column chromatography, with thrombin cleavage of fusion protein. DNA sequence analysis and protein mass spectrometry analysis results were obtained (Research Resources Center, University of Illinois at Chicago). Protein purity was checked with gel electrophoresis, using 16% polyacrylamide gel with 0.1% SDS. Helical contents of the proteins were determined using circular dichroism spectra (Mehboob *et al.*, 2005). We have found that βI-C and βII-C proteins exhibit similar affinities for αII-N (Mehboob *et al.*, 2010). However, βII-C recombinant protein is more

difficult to prepare than βI-C protein due to its low expression level. Thus, βI-C was used for ITC experiments.

ITC measurements were performed at 25 °C using an isothermal titration calorimeter (VP ITC, MicroCal, LLC, Northampton, MA) (Mehboob *et al.*, 2010). Protein pairs (βI-C with αII-N, or αII-N-V22Δ) were dialyzed overnight in 5 mM phosphate buffer with 150 mM sodium chloride at pH 7.4 (PBS) to ensure identical solution conditions in titrating protein pairs. In addition, all samples were thoroughly degassed prior to calorimetry titration. Each αII-N or αII-N-V22Δ sample (30 μM) was titrated into the sample cell containing βI-C protein (3 μM). Titrations of βI-C (30 - 100 μM) into αII-N or αII-N-V22Δ (3 μM) were also performed. Titration isotherms were analyzed with a single binding site assumption, as before (Mehboob *et al.*, 2010), to obtain dissociation constants, K_d.

D. Results

D.1. Yeast Two-Hybrid Assays

For colony growth assay, cells with either βI-C or βII-C and with αII-N or αII-N-V22F, -V22M, or -V22W formed well separated colonies with diameters of 2 - 5 mm after 3 days, with no specific colony size associated with cells of a particular mutant (Figure 7). However, cells with αII-N-V22D, with either βI-C (data not shown) or βII-C (Figure 7) did not show any growth after 3 days (Figure 7).

For the β-galactosidase activity (colony-lift) assay, Y187 cells with αII-N or αII-N-V22F, -V22M, or -V22W showed a distinct blue color, but without a consistent color variation associated with cells with a particular mutation (Figure 8).

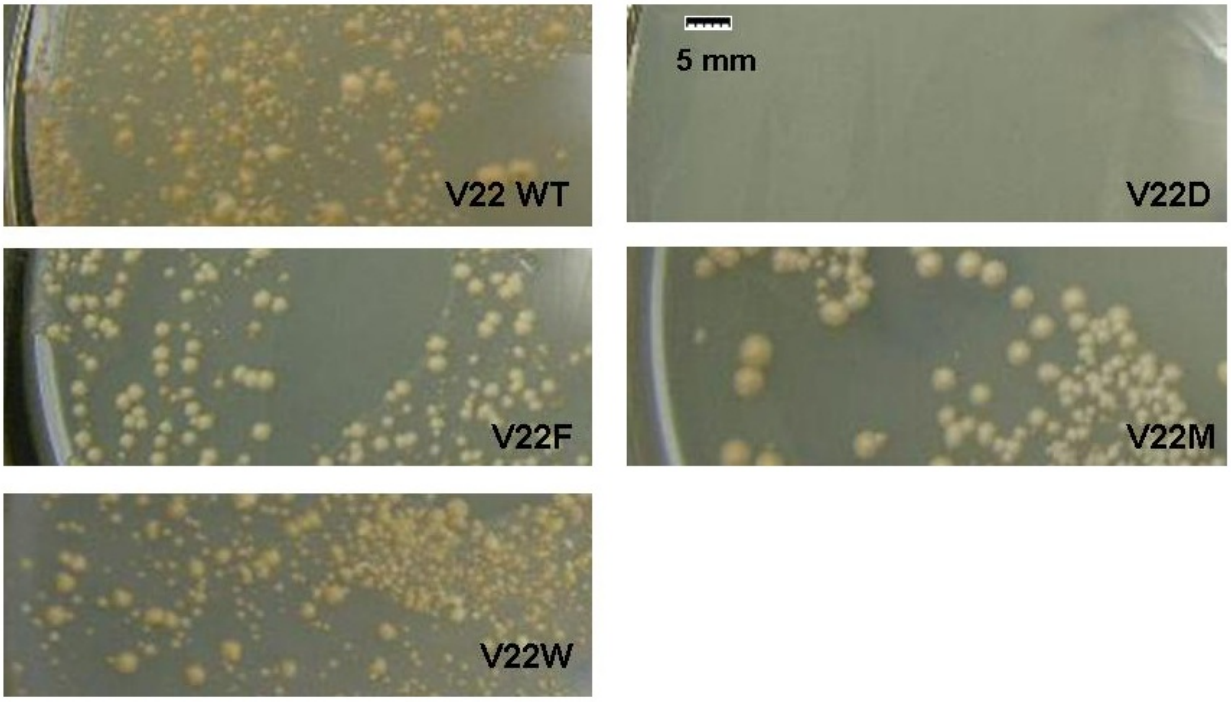

Figure 7. Colony growth assay. AH109 cells co-transformed with pAD-βII-C and pBD-αII-N, or pBD-αII-N-V22D, -V22F, -V22M, or -V22W, were grown for 3 days at 30 °C, following procedures from the manufacturer (Clontech). Colonies, 2 - 5 mm in diameter, were found for cells expressing αII-N (marked as V22 WT above), αII-N-V22F (V22F), αII-N-V22M (V22M), or αII-N-V22W (V22W), whereas cells expressing αII-N-V22D (V22D) did not show any growth. The scale bar is shown in top right panel.

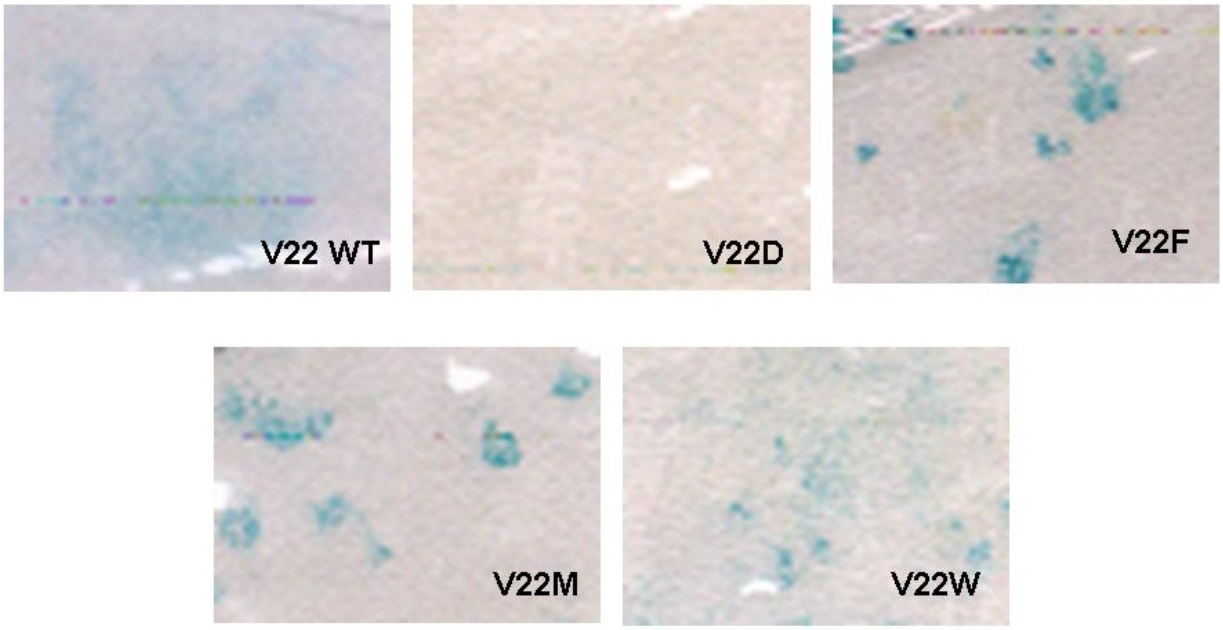

Figure 8. β-Galactosidase activity assay via colony lift method. Y187 cells co-transformed with pAD-βII-C and pBD-αII-N, -αII-N-V22D, -αII-N-V22F, -αII-N-V22M, or -αII-N-V22W were grown for 3 days at 30 °C following procedures from the manufacturer (Clontech). Colonies were transferred onto filter papers, subjected to freeze-thaw cycles, and incubated on a second set of filter papers pre-soaked with β-galactosidase substrate (X-gal) for 30 min. Filter papers for colonies with αII-N, αII-N-V22F, αII-N-V22M and αII-N-V22W all showed blue color, but those with αII-N-V22D did not show blue color.

D.2. Isothermal Titration Calorimetry Assay

D.2.a. Recombinant Protein Analysis

The SDS gel electrophoresis data showed that all αII-N (wild type and mutants) and βI-C proteins were ~90% pure. Electrophoretic masses were ~42 kDa for αII-N proteins and ~22 kDa for βI-C. Mass spectrometric results showed 42,241.0 Da for αII-N (expected mass is 42,242.5 Da), 42,258.6 Da for αII-N-V22D (expected mass is 42,258.5 Da), 42,289.0 Da for αII-N-V22F (expected mass is 42,290.6 Da), 42,274.8 Da for αII-N-V22M (expected mass is 42,274.6 Da), 42,329.8 Da for αII-N-V22W (expected mass is 42,329.6 Da) and 22,036.9 Da for βI-C (expected mass is 22,036.9 Da). The CD spectra of αII-N, αII-N-V22Δ and βI-C exhibited characteristic features of similar spectrin recombinant proteins (Mehboob *et al.*, 2001), with minima at 222 and 208 nm. Helical contents were ~75%, in good agreement with published results (Mehboob *et al.*, 2001).

D.2.b. ITC Results (*done by Marta A. Witek*)

The ITC isotherm of βI-C/αII-N system at 25 °C showed that sufficient heat (-0.45 μcal/sec) was released during titration of αII-N into βI-C (Figure 9), with an average K_d value of 6.9 $\pm$ 0.5 nM (n = 3), in good agreement with previous findings of a similar system (with βI-C) (Mehboob *et al.*, 2003), and the values are similar to that with βII-C (Li and Fung, 2003). The K_d value was 6.7 $\pm$ 0.3 nM for βI-C/αII-N-V22F, 35 $\pm$ 4 nM for βI-C/αII-N-V22M and 93 $\pm$ 28 nM for βI-C/αII-N-V22W. However, for βI-C/αII-N-V22D system, there was insufficient heat released either when αII-N-V22D (30 μM) was titrated with βI-C (3 μM) (Figure 9, Table II), or when βI (30 - 100 μM) was titrated with αII-N-V22D (3 μM), indicating that the K_d value for this system is larger than 100 μM.

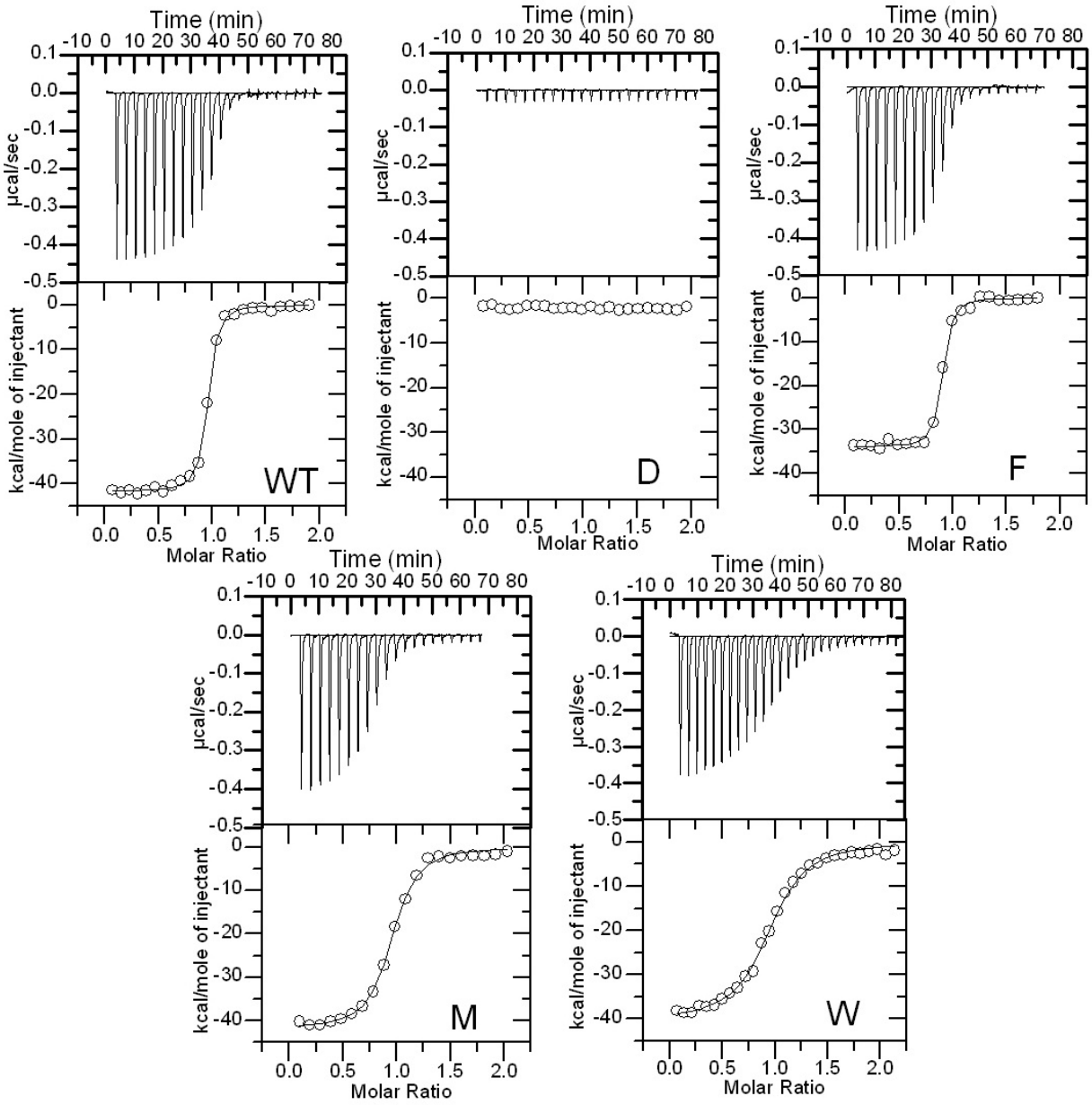
Time (min)
-10 0 10 20 30 40 50 60 70 80
µcal/sec
kcal/mole of injectant
Molar Ratio
WT
D
F
M
W

Figure 9. ITC measurements. Recombinant protein samples of βI-C, αII-N (marked as WT above), αII-N-V22D (D), αII-N-V22F (F), αII-N-V22M (M) and αII-N-V22W (W) were dialyzed together in 5 mM phosphate buffer with 150 mM sodium chloride at pH 7.4 and degassed thoroughly prior to ITC measurements. αII-N proteins (29 - 35 μM) were each individually titrated into the sample cell containing βI-C protein (3 μM). Typical ITC titration isotherms and fitted curves are shown. The average K_d values (n = 3), determined from the fitted curves using a single-binding site model of the manufacturer (MicroCal) software, were 6.9 nM for βI-C/αII-N and 6.7 nM for βI-C/αII-N-V22F, 35 nM for βI-C/αII-N-V22M and 93 nM for βI-C/αII-N-V22W. Little heat was released for βI-C/αII-N-V22D titration and no K_d was obtained.

TABLE II. Y2H and ITC results of alpha and beta spectrin model proteins interaction.

pBD Plasmid[a]	Colony Growth[b]	β-Galactosidase Activity[c]	K_d[d] (nM)
αII-N	Yes	Blue color	6.9
αII-N-V22D	No	No color	not detectable
αII-N-V22F	Yes	Blue color	6.7
αII-N-V22M	Yes	Blue color	35
αII-N-V22W	Yes	Blue color	93

[a]pAD-βII-C with different pBD-αII-N plasmids in the Y2H experiment; we also used pAD-βI-C with pBD-αII-N or pBD-αII-N-V22D, and the results were the same as those with pAD-βII-C; [b]yeast AH109 cells were grown in a medium containing all essential amino acids but tryptophan, leucine and histidine, and lacking adenine; [c]yeast Y187 cells were grown in medium containing all essential amino acids but leucine and tryptophan for the colony lift assay; [d]ITC experiments using recombinant proteins of αII-N and mutants listed and of βI-C were carried out at 25 °C in 5 mM phosphate buffer with 150 mM sodium chloride at pH 7.4.

E. Discussion

The Y2H systems have been widely used to study protein-protein interactions. In this study, both colony growth and β-galactosidase activity detection results showed that αII spectrin with mutations V22F, V22M or V22W interacted with βII spectrin at the tetramerization site (N-terminal region of αII and C-terminal region of βII). However, αII-N-V22D did not interact with βII-C. With those αII mutants that interacted with βII-C, we were not able to detect any differences in interactions between V22F, V22M or V22W with βII-C. Both colony growth rate and colony size, as well as the blue color indication for β-galactosidase activity did not show detectable differences between V22, V22F, V22M and V22W.

The ITC methods require not only the preparation of recombinant proteins but also the characterization of these proteins for proper functional analysis. In our systems, we characterized the protein systems with high resolution mass spectrometry analysis as well as by circular dichroism analysis. We have found that, for both α and β spectrin recombinant proteins used for tetramerization studies, it is important to obtain their CD results to demonstrate that the proteins are folded properly before ITC experiments. The ITC results show that the K_d values for V22F and the wild type with βI-C were about the same, with a K_d of about 7 nM. However, V22M and V22W both exhibited lower affinity than the wild type, with K_d values of 35 nM and 93 nM, respectively. The ITC results of V22D titration with βI-C showed little interaction, with K_d values larger than 100 μM. As indicated in **METHODS**, we have found that βI-C and βII-C proteins exhibit similar

affinities for αII-N (Mehboob *et al.*, 2010), and in this study we showed that results similar to those of βII-C were obtained when βI-C was used with αII-N wild type or with V22D.

Spectrin tetramer formation involves the bundling of three helices, one from α (Helix C') and two from β (Helix A' and Helix B'), forming a triple helical bundle (Mehboob *et al.*, 2010; Mehboob 2001). Mutations that affect the triple helical bundling lead to lower affinity. Previous studies reveal that the V22 position of αII is critical for its tetramerization with βII (Sumandea and Fung, 2005). Sequence alignment shows that αII V22 corresponds to V31 in erythroid α spectrin (αI). αI V31 has been identified as a hot spot that leads to severe clinical symptoms (Lecomte *et al.*, 1993). In triple helical bundling of αII and βII helices, an N-terminal hydrophobic cluster (Li and Fung, 2009) involves three residues in the αII Helix C' (I15, V22, and L23) and two residues in the βII Helix A' (V2019 and F2022), and one residue in the βII Helix B' (F2073) (Mehboob *et al.*, 2010). Thus, it is not surprising that mutations at the V22 position may affect non-erythroid spectrin tetramer formation. Since V22 is involved in a hydrophobic cluster during helical bundling to form tetramers, a mutation from V to a charged residue D clearly weakens the hydrophobic cluster and thus severely reduces the ability of V22D to interact with Helices A' and B' in βII-C. Mutation of V22 to other hydrophobic residues such as V22F did not affect its interaction with βII-C. The mutations of V22M and V22W lowered the affinity by about 5 times and 10 times, respectively. Hydrophobicity of individual side chains, and the properties of the interacting clusters also affected the triple helical bundling. The K_d values determined by ITC represented a ΔG value of about -46.6 kJ/mol (-11.1 kcal/mol) for βI-C with either αII-N or αII-N-V22F, -42.6 kJ/mol (-10.1 kcal/mol)

with αII-N-V22M, and -40.1 kJ/mol (-9.6 kcal/mol) with αII-N-V22W. Thus, the tetramers of these αII mutants and β spectrin exhibit slightly differing stabilities from each other. As discussed previously (Li and Fung, 2009), αII spectrin has recently been reported to be essential for stabilizing nascent sodium channel clusters (Voas *et al.*, 2007), assembling the mature node of Ranvier (Voas *et al.*, 2007), and regulating endothelial cell-cell contacts (Benz *et al.*, 2008). The tetramer formation of αII-βII spectrin is also essential in the regulatory step for neuritogenesis (Bignone *et al.*, 2007). Tetramerization is clearly important for spectrin function. At present, no clinical mutations in αII spectrin, including the tetramerization region, have been identified. A reduced level of spectrin tetramers and abnormal spectrin-based membrane skeleton could cause abnormal neural activities in cells.

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CHAPTER 3

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

This chapter was submitted to "Cellular and Molecular Biology Letters" journal (Sevinc, A., and Fung, L. W.-M.).

A. 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) (Oh and Fung, 2007) 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 binding protein 1 fragment abolished αII-N and βII-C interaction, suggesting that this protein may inhibit or regulate non-erythroid spectrin tetramer formation.

B. Introduction

Spectrin is a cytoskeletal protein, initially identified for its role in preserving the biconcave shape of erythrocyte membranes (Marchesi and Steers, 1968) and originally considered to be present only in erythrocyte (Hiller and Weber, 1977) until the identification of non-erythrocyte isoforms and their functions (Levine and Willard, 1981). Spectrin is involved in the formation and maintenance of plasma membranes at sites of cell-cell contacts (Lee *et al.*, 1993), protein sorting and accumulation (Pinder and Baines, 2000), interactions with structural and regulatory proteins (Djinovic-Carugo *et al.*, 2002), regulation of signal transduction pathways (Gascard and Mohandas, 2000), and regulation of DNA repair (Sridharan *et al.*, 2006). Non-erythroid spectrin (spectrin II), also referred to as brain spectrin (Kanda *et al.*, 1986), calspectin (Tsukita *et al.*, 1983), or fodrin (Sobue *et al.*,1982), is found in neuronal axons (Reiderer *et al.*, 1988), whereas erythroid spectrin (spectrin I) is confined to neuronal cell bodies and dendrites, and some glial cells (Ohara *et al.*, 1988). Beta II spectrin (βII) participates in the propagation of TGF-β signaling (Tang *et al.*, 2003). Gene knock-out studies show that spectrin expression and regulation may not be essential but important for fundamental cellular functions; therefore, spectrin mutations may be non-lethal but cause disease conditions in humans (Bennett and Baines, 2001). The functional form of spectrin is tetrameric (DeSilva *et al.*, 1992), and its 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 (Speicher *et al.*, 1993; Mehboob *et al.*, 2005; Ipsaro *et al.*, 2010; Song *et al.*, 2011). This interaction involves hydrophobic residue clustering, salt bridges and hydrogen bonds (Ipsaro *et al.*, 2010; Song *et al.*, 2011; Antoniou *et al.*, 2008; Song *et al.*, 2009; Mehboob *et al.*, 2010). 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 α-spectrin association affinity between erythroid and non-erythroid spectrin (Mehboob *et al.*, 2001; Mehboob *et al.*, 2003), in good agreement with earlier studies using intact spectrin (Begg *et al.*, 1997). It has been shown that other proteins also interact with the N-terminal region of αII-spectrin (Oh and Fung, 2007). 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.

C. Materials and Methods

C.1. Library Screening for βII-C Interacting Proteins ($IP_{\beta II\text{-}C}$)

The C-terminal region (amino acid residues 1697-2145) of brain (non-erythroid) beta spectrin (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 (Oh and Fung, 2007; Sumandea and Fung, 2005), 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 ^{o}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 ^{o}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 ^{o}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 ^{o}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-$IP_{\beta II-C}$) 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 ^{o}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 ^{o}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 ^{o}C. Those colonies with α-galactosidase production were detected by the appearance of blue colonies as they grew on plates.

C.2. 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 ^{o}C, cells without growth were eliminated, and only those with growth were further analyzed for IP$_{\beta II\text{-}C}$ s.

C.3. 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 ^{o}C.

Once the $IP_{\beta II\text{-}C}$ 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--$IP_{\beta II\text{-}C}$ and pAD-βII-C plasmids to express BD-αII-N and AD-βII-C as well as $IP_{\beta II\text{-}C}$, in the absence of methionine.

In addition to $IP_{\beta II\text{-}C}$ s from this screening, we also studied the effect of αII-N interacting proteins ($IP_{\alpha II\text{-}N}$ s) identified in our earlier screening (Oh and Fung, 2007), on αII-N and βII-C interaction by using a similar experimental set up, replacing $IP_{\beta II\text{-}C}$ with $IP_{\alpha II\text{-}N}$ 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 (Oh and Fung, 2007).

C.4. DNA Sequencing and Protein Identification

Plasmids from positive colonies with $IP_{\beta II\text{-}C}$ 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 SMART III, CDSIII sequences, 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-$IP_{\beta II\text{-}C}$ 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

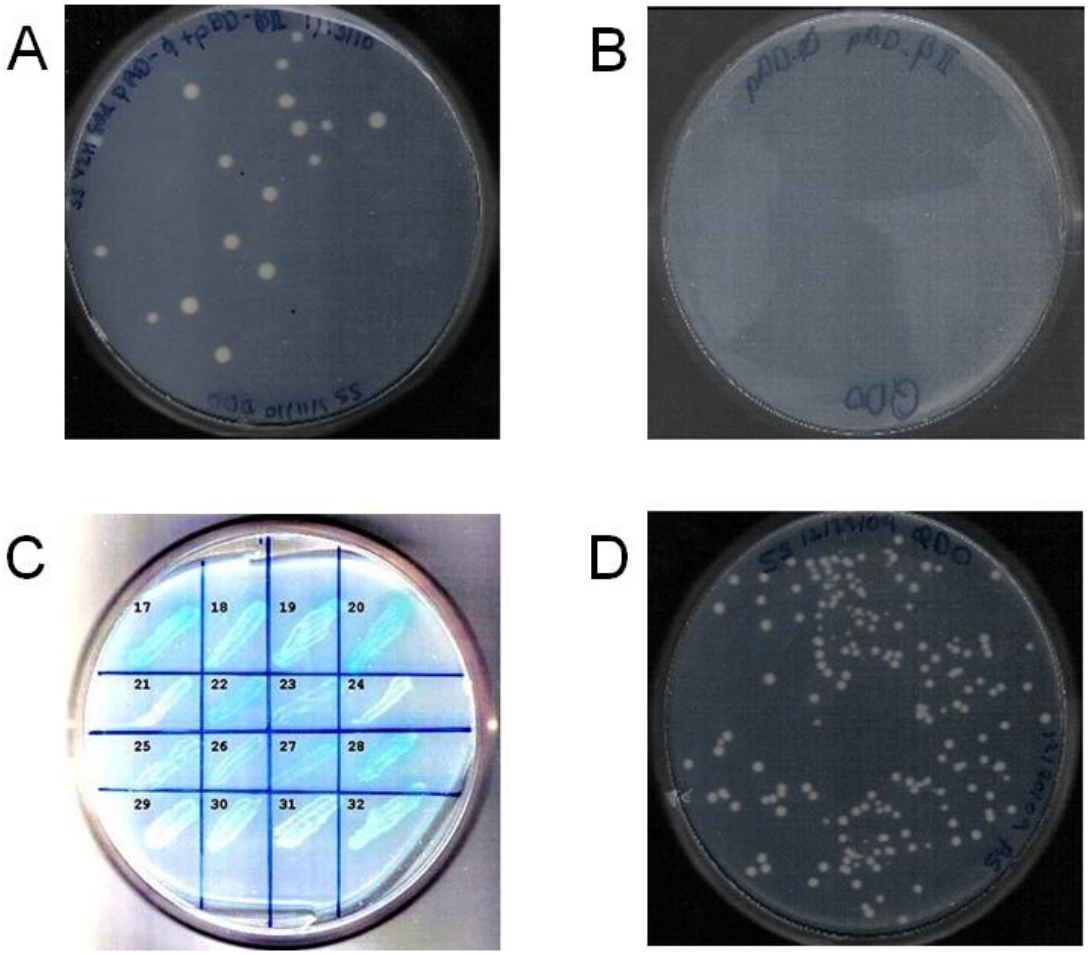
sequences were determined using the Translate tool (ExPASy proteomics server), and the sequences between SMARTIII and the first stop codon was taken as the sequence for the interacting protein. These sequences were analyzed using Blastn, Blastx, and Blastp in "Basic Local Alignment Tool" (http://www.ncbi.nlm.nih.gov/blast/) in all non-redundant BLAST protein sequence databases, as before (Oh and Fung, 2007), for information on the interacting proteins.

D. Results

D.1. 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 (Figure 10A), 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 (Figure 10B), indicating that colony growth is observed only in the presence of an interacting protein X expressed by the pAD-X plasmid.

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 (Figure 10C, for example, colonies on grids 17-20, 22-23, 25-28 and 32) and eliminated 240 of those colonies that appeared as white colonies (Figure 10C, 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 (Figure 10D), confirming that these colonies indeed consisted of proteins that interacted with βII-C.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32
D

Figure 10. 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***).

D.2. 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. Thus, a total of 17 different sequences were obtained from the 20 randomly selected positive colonies (Table III). 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 (Table III, #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. 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 Table III for identification references.

TABLE III. 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).

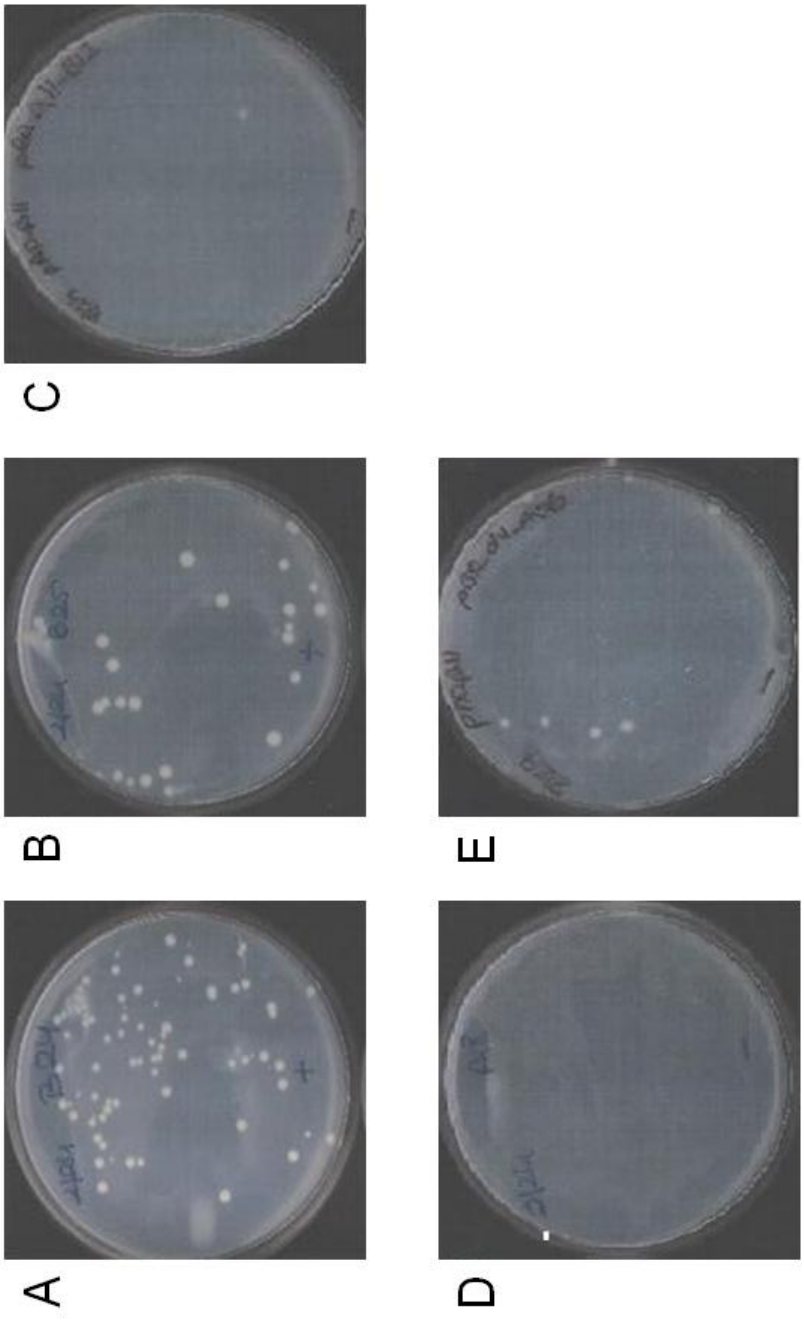
$IP_{\beta II\text{-}C}$	Nucleotides[a]	Frame[b]	Codons[c]	First Three Residues[d]
1	1536	0	**153** (6, 30)	DDD
2	1509	0	**483** (0, 4)	SSF
3	1509	0	**483** (0, 4)	SSF
4	1744	0	**278** (10, 39)	RVG
5	1325	0	**386** (10, 39)	RVG
6	1400	1	303 (7, **49**)	KKK
7	1232	0	**247** (42, 108)	GGS
8	602	0	**131**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

[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.

D.3. αII-N Effect on $IP_{\beta II\text{-}C}$ Interaction with βII-C

The yeast three-hybrid experiments with the cells of the 17 samples grown in the presence of methionine to give AD-$IP_{\beta II\text{-}C}$ and BD-βII-C, but no αII-N show colony growth (Figure 11A), as expected, confirming the interactions between AD-$IP_{\beta II\text{-}C}$ 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-$IP_{\beta II\text{-}C}$ and BD-βII-C, 14 samples showed no colony formation (data not shown), indicating these $IP_{\beta II\text{-}C}$ s did not interact with βII-C in the presence of αII-N. Only the cells with three $IP_{\beta II\text{-}C}$ s ($IP_{\beta II\text{-}C}$-1, -8 and -9) gave colonies (Figure 11B), indicating that $IP_{\beta II\text{-}C}$-1, -8 and -9 interacted with βII-C in the presence of αII-N.

Of these three $IP_{\beta II\text{-}C}$ s that interact with βII-C in the presence of αII-N, only $IP_{\beta II\text{-}C}$-1 showed no colony growth for cells with pBR-αII-N--$IP_{\beta II\text{-}C}$ and pAD-βII-C plasmids, in the absence of methionine (Figure 11C), indicating that the presence of $IP_{\beta II\text{-}C}$-1 abolished the αII-N and βII-C interaction. In the presence of methionine, when no $IP_{\beta II\text{-}C}$-1 was expressed, colonies formed (data not shown).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B
E
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Figure 11. Effects of interacting proteins on spectrin tetramerization.

AH109 cell colonies with yeast three-hybrid plasmids (pAD-$IP_{\beta II-C}$-1 and pBR-βII-C--αII-N for ***A*** and ***B***, pAD-βII-C and pBR-αII-N--$IP_{\beta II-C}$-1 for ***C***, pAD-$IP_{\alpha II-N}$-18 and pBR-αII-N--βII-C for ***D*** and, pAD-βII-C and pBR-αII-N--$IP_{\alpha II-N}$-6 for ***E***), after 3 days of growth at 30 °C on two different types of medium, with or without methionine, to understand αII-N effect on $IP_{\beta II-C}$ interaction with βII-C (***A*** - ***C***), and βII-C effect on $IP_{\alpha II-N}$ interaction with αII-N (***D*** - ***E***). Cells grown in the presence of methionine, consisting of AD-$IP_{\beta II-C}$-1 and BD-βII-C showed colony growth (***A***). Only cells with $IP_{\beta II-C}$-1, -8 and -9 showed colony growth when grown in the absence of methionine, consisting of AD-$IP_{\beta II-C}$, BD-βII-C and αII-N (***B***), whereas the rest of the samples did not (results not shown). This indicates that, 3 $IP_{\beta II-C}$ s that showed colony growth were able to bind βII-C in the presence of αII-N. When one of these 3 $IP_{\beta II-C}$ s were expressed in cells consisting of AD-βII-C and BD-αII-N, colony growth was not observed (***C***). This indicates that in the presence of that $IP_{\beta II-C}$, αII-N and βII-C were not able to interact. Cells grown in the presence of methionine, consisting of AD-$IP_{\alpha II-N}$ and BD-αII-N showed colony growth (results not shown). Cells with 4 $IP_{\alpha II-N}$ s showed colony growth when grown in the absence of methionine, consisting of AD-$IP_{\alpha II-N}$, BD-αII-N and βII-C (results not shown), whereas the rest of the samples did not (***D***). Colony growth was observed when cells with plasmids for $IP_{\alpha II-N}$-6 grown in the presence of methionine, expressing AD-βII-C and BD-αII-N, but not $IP_{\alpha II-N}$ (results not shown), as well as in the absence of methionine, expressing AD-βII-C and BD-αII-N, and $IP_{\alpha II-N}$-6 (***E***). This indicates that the presence of $IP_{\alpha II-N}$-6 do not interfere with αII-N / βII-C interaction.

D.4. Alignment of βII-C Interacting Proteins 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" (Table IV). 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) (Table IV). 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 (Table IV).

$IP_{\beta II\text{-}C}$ -4 was similar to zinc-finger protein 251 (residues 146-614) (2% difference), and $IP_{\beta II\text{-}C}$ -1 was similar to syntaxin binding protein 1 (residues 284-435) (4% difference). The remaining proteins exhibited lower homology values to known proteins and the last 5 proteins did not match the sequences of any known proteins (Table IV).

TABLE IV. βII-C interacting proteins ($IP_{βII-C}$ s) and their effects on tetramerization site interaction.

$IP_{βII-C}$	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 (AAY24229), 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	1-150* 151-168* 169-247* (303)	Golgin A6 family-like 10 (NP_001157937), residues 47-196; 211-228; 243-321 (479)	26% *I4V, E39Q, R156H, R163C, Q169L, D174E, R191C, E237D	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 $IP_{βII-C}$ and βII-C interaction, with "yes" indicating that αII-N abolishes $IP_{βII-C}$ interaction with βII-C; [b]effects of $IP_{βII-C}$ on αII-N and βII-C interaction; [c]total number of residues in protein; [d]the symbol * indicates mutations exist.

D.5. 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 similar to Figure 3.2B, indicating that these $IP_{\alpha II\text{-}N}$ (TBP-associated factor, lysyl-tRNA synthetase, and two fragments of Zinc finger protein 333 (1-169 residues and 1-230 residues fragments) interacted with αII-N in the presence of βII-C. Cells with the remaining 3 $IP_{\alpha II\text{-}N}$ s did not show any colony growth (Figure 3.2D).

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 (Figure 3.2E).

E. 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 (Mehboob *et al.*, 2005; Ipsaro *et al.*, 2010; Song *et al.*, 2009). 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 mM (Mehboob *et al.*, 2003). 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 (Oh and

Fung, 2007). 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 (Marchler-Bauer *et al.*, 2011), 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* (Roussigne *et al.*, 2003). Another βII-C interactor ($IP_{\beta II\text{-}C}$-4) also binds zinc ion and is identical to a fragment (residues 146-416) of zinc finger protein 251 (UniProt: 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 (Oh and Fung, 2007). 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. $IP_{\beta II\text{-}C}$-5 is also similar to zinc finger protein 251 (residues 317-671), but with numerous mutations.

$IP_{\beta II\text{-}C}$-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 ($IP_{\beta II\text{-}C}$-3) that consists of the first 258 residues and residues 283-478 of this protein. $IP_{\beta II\text{-}C}$-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 (Macara *et al.*, 2002; Peterson and Petty, 2010). The 6 $IP_{\beta II\text{-}C}$ 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.

$IP_{\beta II\text{-}C}$-9, identical to the C-terminal 250 residues of coatomer subunit beta (residues 704-953) except with one mutation (Y53 in $IP_{\beta II\text{-}C}$-9 and 756C in coatomer subunit beta), interacts with βII-C even in the presence of αII-N, suggesting strong affinity with βII-C. Similar to $IP_{\beta II\text{-}C}$-9 in affinity are $IP_{\beta II\text{-}C}$-1 and $IP_{\beta II\text{-}C}$-8. $IP_{\beta II\text{-}C}$-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 (Han *et al.*, 2010). $IP_{\beta II\text{-}C}$-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 (David *et al.*, 2010; Ardley *et al.*, 2000). With these three strongly interacting proteins, only $IP_{\beta II\text{-}C}$-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 $IP_{\beta II\text{-}C}$ 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 (Good *et al.*, 2011). 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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CHAPTER 4

$IP_{\beta II-C}$-1 PURIFICATION

A. Introduction

Tetramerization is an important feature of spectrin isoforms for performing their fundamental cytoskeletal functions, which involves helical bundling of the single helix (Helix C') at the N-terminal region of α spectrin, and two helices (Helix A' and Helix B') at the C terminal region of β spectrin. Our previous studies have identified several mutations on the N-terminal region (residues 1 – 359) of brain α spectrin (αII-N) effecting spectrin tetramerization (Sumandea and Fung, 2005), and several proteins interacting with αII-N at the tetramerization site (Oh and Fung, 2007).

Using yeast library screening methods, we identified 17 different proteins that interact with the C-terminal region (residues 1697 – 2145) of brain β spectrin (βII-C) in a human brain cDNA library. Three of these proteins ($IP_{\beta II-C}$-1, $IP_{\beta II-C}$-8, and $IP_{\beta II-C}$-9) were able to bind to βII-C even in the presence of αII-N, suggesting that these proteins exhibit a higher affinity for βII-C than αII-N. Furthermore, $IP_{\beta II-C}$-1 was shown to abolish the interaction between αII-N and βII-C, which implicates that it may be involved in regulation of the interaction of αII-N and βII-C, spectrin tetramerization, and in turn cytoskeletal organization. Detailed studies on the interaction between $IP_{\beta II-C}$-1 and βII-C will provide mechanistic understanding of these interactions. Assembly and disassembly of components of the submembranous cytoskeleton including αII and βII spectrin may regulate the formation of the SNARE complex (Nakano *et al.*, 2001).

Sequence alignment studies showed that $IP_{\beta II-C}$-1, except for the last 8 residues, residues 1-145 are identical to a fragment (residues 284 – 428) of "syntaxin binding protein 1", a protein closely related to a group of proteins that has been previously shown to interact with spectrin isoforms (Nakano *et al.*, 2001).

Syntaxin binding protein 1, (also known as p67, unc18, Munc 18) was first discovered as a partner of the SNARE complex interacting with syntaxin 1a with nanomolar affinity (Pevsner *et al.*, 1994; Hata *et al.*, 1993; Rickman *et al.*, 2007) and indirectly interacting with cytoskeleton (Nakano *et al.*, 2001; Khanna *et al.*, 2007). Syntaxin binding protein 1 has been shown to be essential for regulated exocytosis, most probably through regulating the SNARE-assembly reaction (Lang and Jahn, 2008). Cellular functions and location of syntaxin binding protein 1 appears to be closely related to syntaxin 1a, and syntaxin 1a has been shown to interact with αII spectrin (Nakano *et al.*, 2001). Over expression of this protein leads to a decrease in neurotransmitter release (Schulze *et al.*, 1994), whereas null alleles and certain point mutations lead to a block in vesicle fusion (Schekman, 1992; Verhage *et al.*, 2000), and this indicates that syntaxin binding protein 1 (and its orthologs in other species, such as yeast, *Drosophila*) not only maintain and regulate the SNARE complex at the sites of exocytosis, but also assist the syntaxin proteins in adopting a functional conformation or facilitate interactions of the SNARE complex proteins and other proteins. This chaperone like activity of syntaxin binding protein 1 proteins was also shown by the failure of the transport of syntaxin 1a to the plasma membrane from the golgi apparatus (Rowe *et al.*, 1999). And finally, it has been shown that syntaxin binding proteins also interacts with RAB proteins, which are small GTPases that are essential for vesicle trafficking and membrane fusion (Pfeffer, 1999).

It has been suggested that the spectrin network formation beneath the plasma membrane may function as a barrier to prevent vesicles from docking with the plasma membrane (Perrin and Aunis, 1985). Therefore the dissolution of the cytoskeleton prior to the docking of the vesicle may be enhanced by the dissociation of αII-spectrin from syntaxin family members, which may induce the formation of the SNARE complex. At this point, syntaxin binding protein 1 may act as an accelerator for the formation of the SNARE complexes by binding to the syntaxin protein and the simultaneous dissolution of the spectrin cytoskeleton.

Crystal structure of the syntaxin binding protein 1 bound to syntaxin 1a has been shown at 2.6 Å resolution (Misura *et al.*, 2000) (Figure 12). Syntaxin binding protein 1 consists of 3 domains; domain 1 formed by residues 4 – 134, domain 2 by residues 135 – 245 and 480 – 592, and domain 3 by 246 – 479. Close examination of the structure reveals that the $IP_{\beta II\text{-}C}$-1 fragment matches to a considerably large portion of Domain 3 (residues 284-428) (Figure 12), which is located at the opposite side of the region that is involved in its interactions with the syntaxin and the SNARE complex (Dubulova *et al.*, 2003; Ciufo *et al.*, 2005; Latham *et al.*, 2006; Dulubova *et al.*, 2007) (Figure 12). It is interesting to note that, the third domain shows structural similarity to a variety of helical repeat proteins (Misura *et al.*, 2000). It is mainly made up of a series of alpha helices that is kinked at a non-helical glycine residue, between helices 12 and 13, separating this domain into two sub-domains, 3a (residues 241 – 359) and 3b (residues 362 – 460). Domain 3a does not show much interaction with the rest of the protein, whereas domain 3b does (Misura *et al.*, 2000).

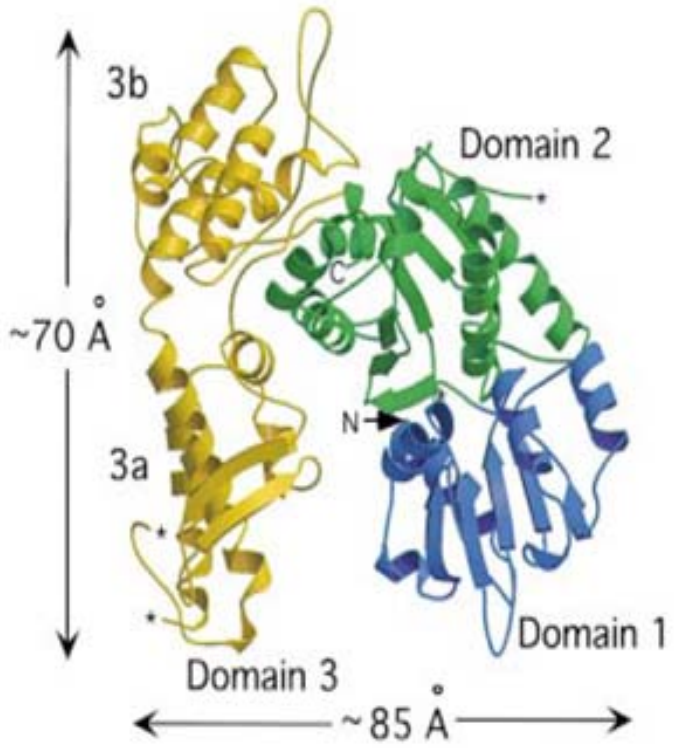


Figure 12. Structure of syntaxin binding protein 1. Ribbon presentation of syntaxin binding protein 1 (Adapted from Misura *et al.*, 2000). The conformation of the protein is as it appears in complex with Syntaxin 1a, which is available (PDB code 3c98).

B. Methods Used for $IP_{\beta II\text{-}C}$-1 Preparation

B.1. Cloning of $IP_{\beta II\text{-}C}$-1 into Bacterial Expression Plasmid, pGEX-2T

The cDNA of $IP_{\beta II\text{-}C}$-1 was transferred from the library plasmid, pGAKT7 (pAD), to bacterial expression plasmid, pGEX-2T, using standard laboratory procedures for PCR amplification (primers used are presented in appendix A), restriction enzyme digestion, ligation and transformation to give pGEX-$IP_{\beta II\text{-}C}$-1 in *E. coli* DH5α cells. DNA sequence analysis of the prepared plasmid was done at the Research Resources Center, University of Illinois at Chicago.

B.2. Expression and Purification of $IP_{\beta II\text{-}C}$-1

Recombinant GST-$IP_{\beta II\text{-}C}$-1 fusion protein was prepared, following standard laboratory techniques (Mehboob *et al.*, 2010) with minor modifications. Briefly, BL21 Codon+ cells were transformed with pGEX-$IP_{\beta II\text{-}C}$-1 and analyzed for isopropyl-beta-D-thiogalactopyranoside (IPTG) induced expression of the fusion protein by whole cell electrophoresis in order to determine the conditions for optimum cell growth and protein expression. An overnight culture (0.5 mL, LB medium with ampicillin) was used to inoculate LB or TB medium (7 mL, with ampicillin) and incubated 3 hours at 37 °C with shaking (240 rpm). This was followed by induction of recombinant protein expression by addition of 5 - 10 mM IPTG to the cell culture, and incubation for another 3 hours at 30 or 37 °C, taking samples 3 hours of protein expression analysis. Upon determination of the optimum conditions for growth and recombinant protein expression, mini-expression analysis of the protein expression was performed following the standard lab procedures, in order to quantitate the fusion protein production.

For large scale growth, a freeze-down vial of BL21 Codon+ cells were used to inoculate 200 mL LB medium containing ampicillin for overnight growth at 37 °C, in an auto-shaker at 240 rpm, and this cell culture was added to a high density fermenter BioFlo (New Brunswick Scientific Co, Inc.) containing 2 L TB medium with ampicillin and 1 mL antifoam, and allowed to grow for 3 h at 37 °C with supplementary air. 5 mM IPTG for 2 L growth in fermenter was added, and cells were incubated for another 3 hours at 30 °C. Cells were harvested and were either directly used for protein purification or stored at -80 °C.

Cells were suspended in 1% Triton in PBS 7.4, disrupted by sonication for 15 min, and centrifuged at 18,000 g for 12 min. Supernatant containing fusion protein was collected and passed through a glutathione-affinity column (glutathione-Sepharose 4B from Pharmacia) to allow GST fusion protein to bind to the resin. The column was extensively washed with PBS 7.4 before eluting the fusion protein with buffer with glutathione (γ-Glu-Cys-Gly). Fusion protein fractions (5 mL) were collected and 10 μL sample was analyzed on 16% polyacrylamide gel with 0.1% SDS (16% SDS-PAGE). The molecular mass was determined at Proteomics Center at the Research Resources Center at University of Illinois at Chicago.

To prepare pure protein (recombinant protein without the N-terminal GST tag), thrombin (50 unit/μmole of fusion proteins) digestion was performed to cleave the GST tag at the engineered recognition site in a TbR buffer and removal of the cleaved N-terminal GST tag by affinity column chromatography to collect pure protein. Our initial attempts to prepare pure $IP_{\beta II\text{-}C}$-1 were hampered by the precipitation of the protein products after the thrombin reaction. Therefore, we did not continue our standard procedure, and used the GST fusion of the $IP_{\beta II\text{-}C}$-1 protein in our analysis. Fusion protein was frozen in liquid nitrogen and stored at -80 °C for further characterization.

Protein concentrations were determined by UV spectrophotometer at 280 nm using an extinction coefficient values of 55,810 M^{-1} cm^{-1} as determined from sequence using the ProtParam tools from ExPASy.

B.3. $IP_{\beta II-C}$-1 Fusion Protein Solution Property

Solution property of the fusion protein was analyzed using size-exclusion chromatography. A Sephacryl(R) 10-HR column (GE Healthcare, Piscataway, NJ) was packed following manufacturer's instructions. The void volume (V_0) of the prepared column was determined by measuring the elution volume of the Blue Dextran (Sigma Aldrich, St. Louis, MO), with a molecular mass of 2,000,000 Da. The column was calibrated with protein samples of known molecular masses (Ribonuclease A, 13,700 Da; Carbonic Anhydrase, 29,000 Da; Ovalbumin, 43,000 Da; and Conalbumin, 75,000 Da) with a flow rate of 72 mL per hour (1.2 mL/min). A calibration curve was plot with natural logarithm of the molecular mass of the calibration proteins versus the calculated phase distribution coefficient (K_{av}) values. K_{av} for each protein was calculated using the elution volume (V_E) of the protein. V_E of a protein is proportional to the percentage of the column volume accessible to the protein due to its molecular size and the porous structure of the column. In order to use this correlation we use the phase distribution coefficient (K_{av}) value, which is defined as the ratio of the pore volume of the column that is accessible to the substance to the total pore volume of the column, as in $K_{av} = (V_E - V_0) / (V_t - V_0)$, where V_E elution volume of the protein, V_0 is the void volume of the mobile phase, V_t is the total liquid volume of the column.

Finally, 5 mL sample of $IP_{\beta II-C}$-1 fusion protein with A_{280} of 3.23 was passed through the column using similar flow rate (73 mL per hour), and the elution profile was recorded. Elution volume (V_E) of each protein was measured, and the K_{av} value was calculated. Using the K_{av} value and the calibration curve, a corresponding molecular mass of each species in solution was obtained.

C. Results

C.1. Cloning of $IP_{\beta II-C}$-1 into *E.coli* Expression Plasmid, pGEX-2T

Agarose gel electrophoresis analysis of the double restriction enzyme digestion product, showed the presence of two bands, one of which had a calculated size of 6.0 kbp, which is close to the expected size of the pGEX-2T plasmid (6,024 bp), and the second band with a calculated size of, ~450 bp, which is considerably close to the expected size of the PCR product (462 bp), coding for the library protein $IP_{\beta II-C}$-1 (Figure 13, middle lane). DNA sequence results showed the presence of the full length GST sequence, thrombin cleavage site, and the library sequence $IP_{\beta II-C}$-1 sequence (Figure 14).

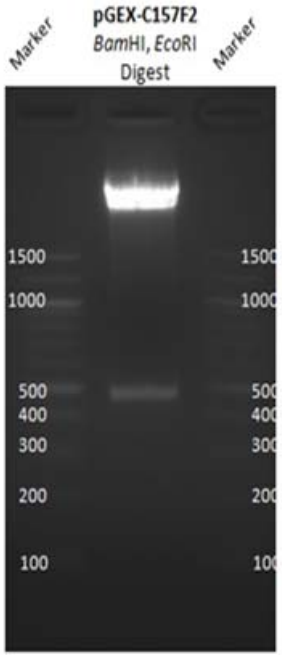


Figure 13. Cloning of GST-$IP_{\beta II\text{-}C}$-1. GST-$IP_{\beta II\text{-}C}$-1 sequence was cloned into bacterial expression vector, pGEX-2T, cleaved by *Bam*HI and *Eco*RI restriction enzyme digestion. The center lane shows the digestion product sample bands, with the bright band at ~6 kbp, which corresponds to expected size of the empty pGEX-2T plasmid, and the other band at ~450 bp, which corresponds to expected size of the $IP_{\beta II\text{-}C}$-1 sequence, indicating the presence of the sequence in the plasmid.

GST $IP_{\beta II-C}$-1 fusion protein DNA sequence

GTGGATGTGAGCGGATACAATTTCACACAGGAAACAGTATTCATGTCCCCTATACTAGGTTATTGGAAAA

TTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTGAAGAAAAATATGAAGAGCATTTGTA

TGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCT

TATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACA

ACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAG

ATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTA

CCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCC

ATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTT

CCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAATCCAGC

AAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGG

ATCTGGTTCCGCGTGGATCCGGGGACGACGACCTGTGGATAGCACTGCGCCACAAGCACATCGCAGAGGT

*Bam*HI

GTCCCAGGAAGTCACCCGGTCTCTGAAAGATTTTTCTTCTAGCAAGAGAATGAATACTGGAGAGAAGACC

ACCATGCGGGACCTGTCCCAGATGCTGAAGAAGATGCCTCAGTACCAGAAAGAGCTCAGCAAGTACTCCA

CCCACCTGCACCTTGCTGAGGACTGTATGAAGCATTACCAAGGCACCGTAGACAAACTCTGCCGAGTGGA

GCAGGACCTGGCCATGGGCACAGATGCTGAGGGAGAGAAGATCAAGGACCCTATGCGAGCCATCGTCCCC

ATTCTGCTGGATGCCAATGTCAGCACTTATGACAAAATCCGCATCATCCTTCTCTACATCTTTTTGAAGA

ATGGCATCACGGAGGAAAACCTGAACAAACTGATCCAGTCTAAGTCACAGTGCCCTGACAGGTGAGAATT

*Eco*RI

CATCGTGACTGACTGACGATCTGCCTCGCGCGTTTACGGGAGACATA

Figure 14. Sequence analysis of pGEX-$IP_{\beta II-C}$-1. Sequence coding for GST is highlighted, restriction enzyme recognition sites are underlined and labeled, and sequence coding for $IP_{\beta II-C}$-1 is written in bold.

C.2. Expression and Purification of $IP_{\beta II-C}$-1

The expected mass from the sequence of GST-$IP_{\beta II-C}$-1 fusion protein was 44,063 Da. The gel (Figure 15, lanes 3 and 4) showed a clear increase in the intensity of the band at 44 kDa upon addition of IPTG. Mass spectrometry results showed one species at 44,062.5 Da (Figure 16). Mini-expression analysis of the culture grown with the determined conditions verified the presence of the $IP_{\beta II-C}$-1 fusion protein in our cells, and that considerable amount of the protein (1.5 mg per g of cells) was able to bind glutathione affinity column via their GST fusion tags (Figure 17, lane 4).

10 μg samples from several preparation of the $IP_{\beta II-C}$-1 fusion protein was analyzed on an SDS-PAGE gel, and analysis of the band intensities showed that the fusion protein was at least 80% pure (Figure 18), with minor bands at 30, 32, 34 kDa (Table V).

Based on the results summarized for the expression and purification of $IP_{\beta II-C}$-1 fusion protein, we concluded that we have accumulated about 186.1 mg of fusion protein samples (Table V). Different preparation dates and the amounts prepared are tabulated in Table V and the SDS electrophoresis these samples are shown in Figure 19. Samples with purity above 47 % has been mixed, and 120.6 mg of fusion protein mix has been prepared to be used for further analysis. Using the measured purity of each preparation, total amount of fusion protein in each sample was calculated, and this shows that the final mix had 80.4 mg of fusion protein.

To remove the GST tag, thrombin was added to GST fusion protein with the A_{280} of the solution around 1.0. Upon completion of the thrombin reaction protein precipitates were found on the surface of the solution. Keeping the solution on ice, lowering the protein concentration (to A_{280} of 0.25) or buffer composition (i.e. using PBS instead of TbR) did not show any improvement in the solubility of the pure protein.

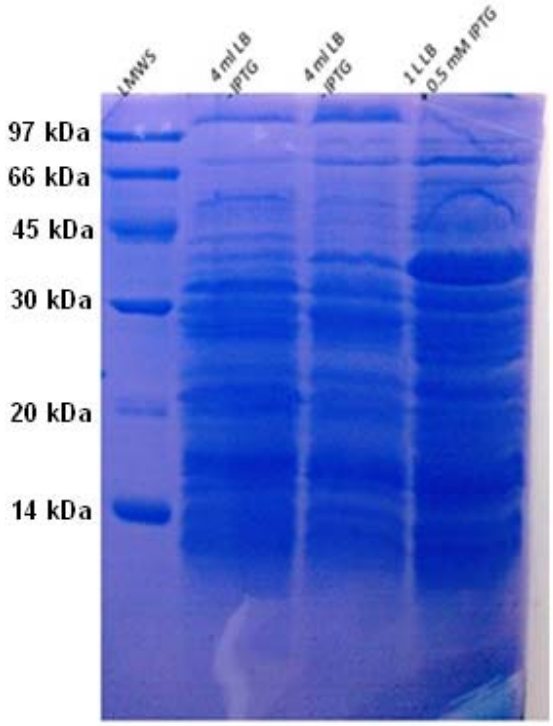


Figure 15. Whole cell electrophoresis analysis of GST-IP$_{\beta II\text{-}C}$-1. Confirmed sequence was transformed into *E.coli* cells BL21 Codon+, and used in whole cell electrophoresis to check over-expression of fusion protein.

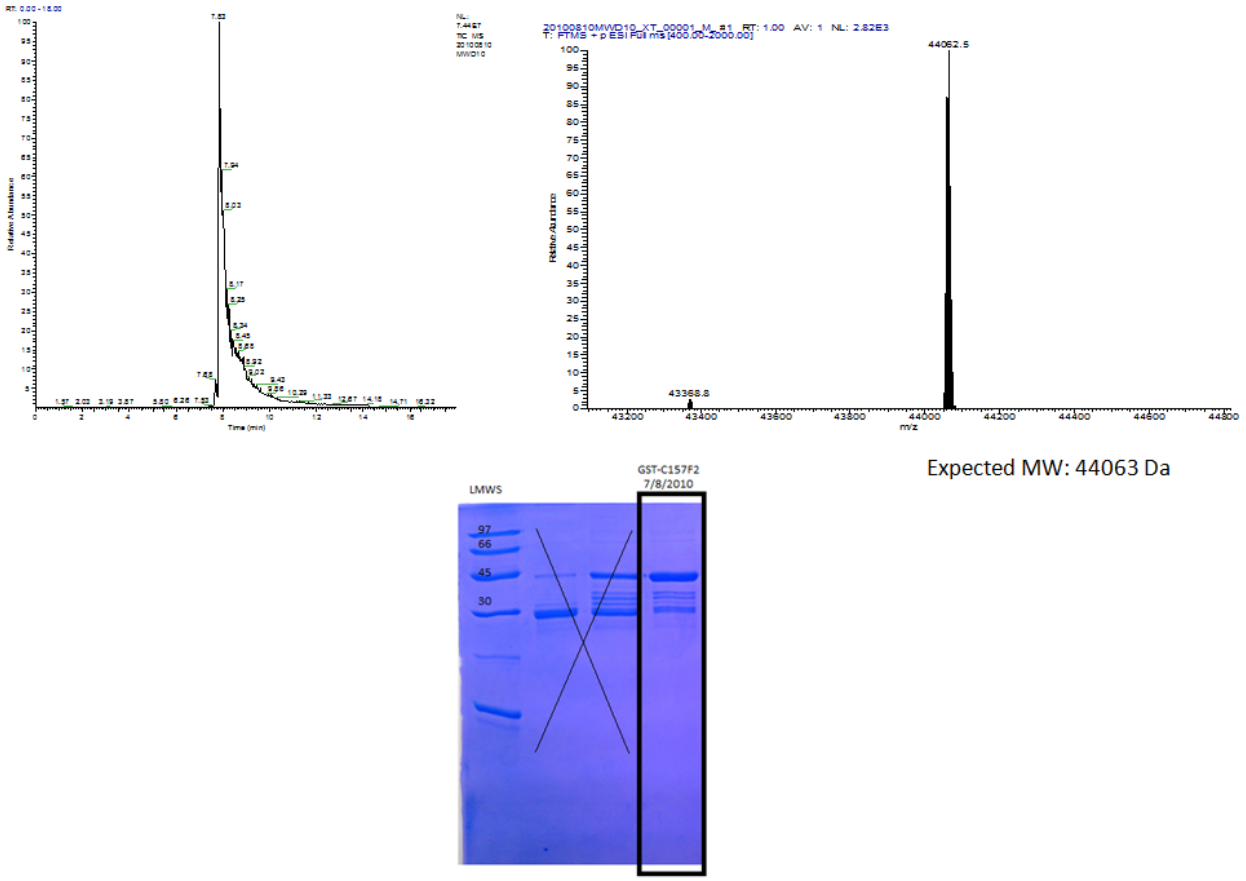


Figure 16. Mass spectrometry analysis of the $IP_{\beta II\text{-}C}$-1 fusion protein. Prepared fusion protein was analyzed by mass spectrometry, and the results are depicted in this figure. Elution profile of the sample (A), mass spectroscopy analysis of a elution time range including the major peak of the elution (B), and the SDS-Electrophoresis analysis of the sample is presented. Elution profile shows the presence of one predominant peak, mass spectroscopy analysis shows the presence of a species with molecular mass of 44,062.5 Da – which is 0.001% different from the expected value of 44,063 – well below the accuracy (0.01%) of the instrument (RRC, Proteomics and Informatics Services Facility), whereas, the SDS gel shows the presence of several other minor species with a molecular weight smaller than the predominant protein species.

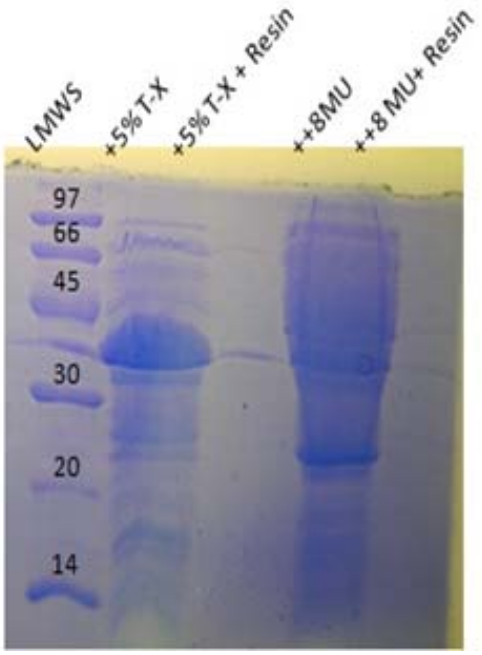


Figure 17. Large scale preparation of GST-$IP_{\beta II\text{-}C}$-1. Upon determining the proper conditions for production of high yields of fusion protein, large scale cell growth was performed and fusion protein was purified. First lane is the low molecular weight standard that is composed of proteins with known molecular masses (97 kDa, 66 kDa, 45 kDa, 30 kDa, 20 kDa, and 14 kDa). Following lanes has the mini-expression analysis results of the GST-$IP_{\beta II\text{-}C}$-1 protein. BL21 codon+ cells containing the bacterial expression plasmid for the recombinant protein is grown with conditions outlines in the whole cell electrophoresis experiments. Known amounts of cells were used to solubilize cellular proteins (5 µL sample is in lane 2) using 5% Triton X-100. This solution was mixed with glutathione resin and bound fusion protein was separated from the beads and was analyzed (5 µL sample is in lane 3). Finally, to check the presence of insoluble fusion protein in the cell debris created in the first step, the cell pellet was dissolved using a stronger detergent (8 M urea), was analyzed (5 µL sample is in lane 4), and any fusion protein bound to glutathione resin was analyzed (5 µL sample is in lane 5).

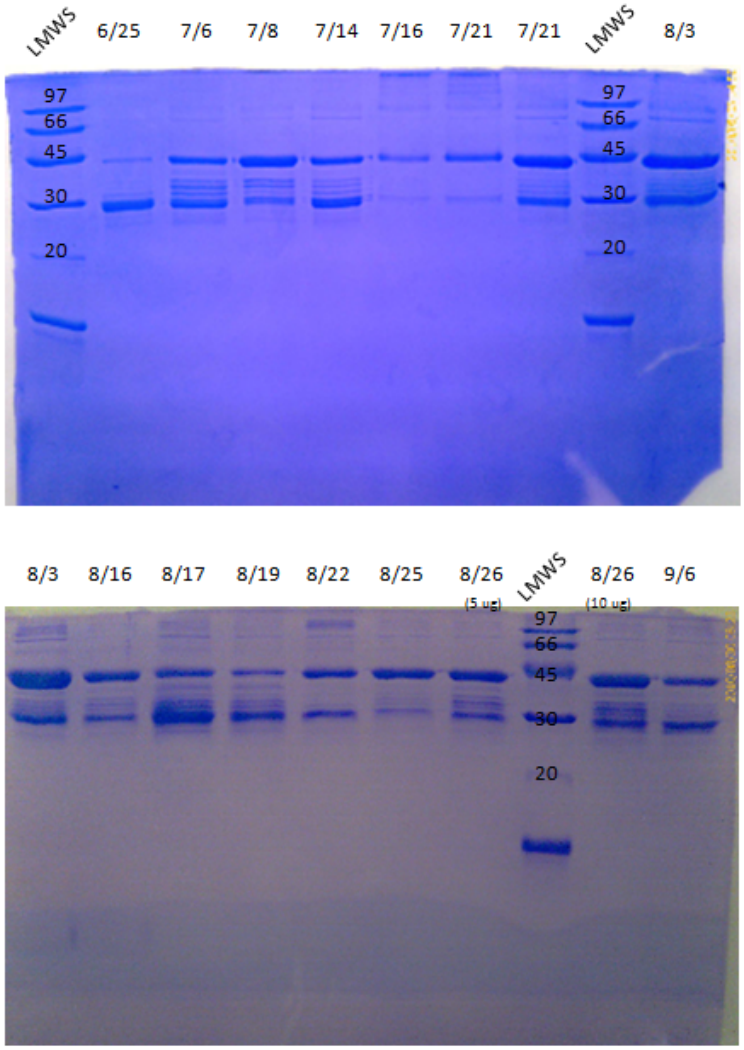


Figure 18. SDS electrophoresis results of all the $IP_{\beta II\text{-}C}$-1 fusion protein samples.

TABLE V. $IP_{\beta II\text{-}C}$-1 fusion protein amounts and purities in each preparation. Samples showing a purity of 47% or lower were not selected (labeled as "Not included") and those selected were mixed to form a homogeneous mixture sample.

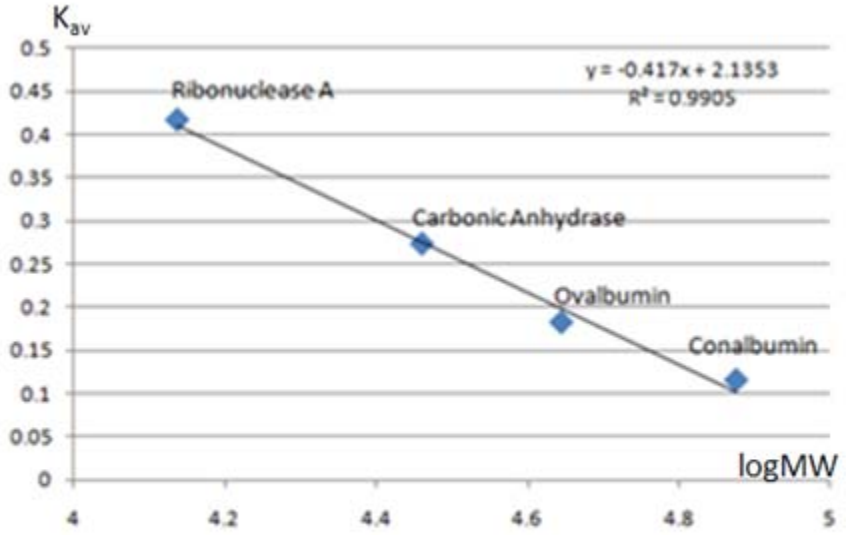
Preparation Date	A_{280}	Volume (mL)	Amount (mg)	Purity	Amount of Fusion Protein
7/6/2010	1	25	25	35%	Not included
7/8/2010	0.6	20	12	52%	6.2 mg
7/14/2010	0.5	16	8	35%	Not included
7/16/2010	0.2	7.5	1.5	77%	1.2 mg
7/21/2010	0.25	22	5.5	66%	3.6 mg
7/21/2010	0.25	28	7	66%	4.6 mg
8/3/2010	0.8	25	20	53%	10.6 mg
8/16/2010	0.9	30	27	57%	15.4 mg
8/17/2010	0.35	30	10.5	23%	Not included
8/19/2010	0.9	6	5.4	24%	Not included
8/22/2010	0.6	25	15	59%	8.9 mg
8/25/2010	0.75	30	22.5	71%	16.0 mg
8/26/2010	0.5	30	15	55%	8.25 mg
9/6/2010	0.65	18	11.7	48%	5.6 mg
		Total	186.1 mg	Total Mix	80.4 mg

C.3. Size Exclusion Chromatography

Sephacryl(R) 10-HR column was packed according to the manufacturer's instructions, and the total liquid volume of the column (V_t) was measured as 199 mL. The void volume of the mobile phase (V_0) was found as 73.8 mL. The elution volume (V_E) for Conalbumin (molecular mass: 75,000 Da, log MW: 4.875) was 88.2 mL to give a K_{av} of 0.1150. Similarly, the V_E for Ovalbumin (molecular mass: 44,000 Da, log MW: 4.644) Carbonic anhydrase (molecular mass: 29,000 Da, log MW: 4.46), Ribonuclease A (molecular mass: 13,700 Da, log MW: 4.1367), were 96.6 mL, 108 mL, and 126 mL, respectively, which to give K_{av} values of 0.1592, 0.2732, 0.4169, respectively. The calibration plot for this column is presented in Figure 19A.

Next, 16 mg of GST-$IP_{\beta II\text{-}C}$-1 fusion protein was passed through the column (Figure 19B) and two peaks were observed at fraction number 28 (V_E: 82.5 mL) and fraction number 34 (V_E: 100.5 mL). Our calculations showed that these elution volumes corresponded to K_{av} values of 0.0695 and 0.2133, and molecular masses of 89,949 Da and 40,654 Da for the protein samples obtained in two peaks. This indicated that the $IP_{\beta II\text{-}C}$-1 fusion protein in solution exists as monomers and dimers.

A


K_{av}
y = -0.417x + 2.1353
R^2 = 0.9905
Ribonuclease A
Carbonic Anhydrase
Ovalbumin
Conalbumin
logMW
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
4
4.2
4.4
4.6
4.8
5

B

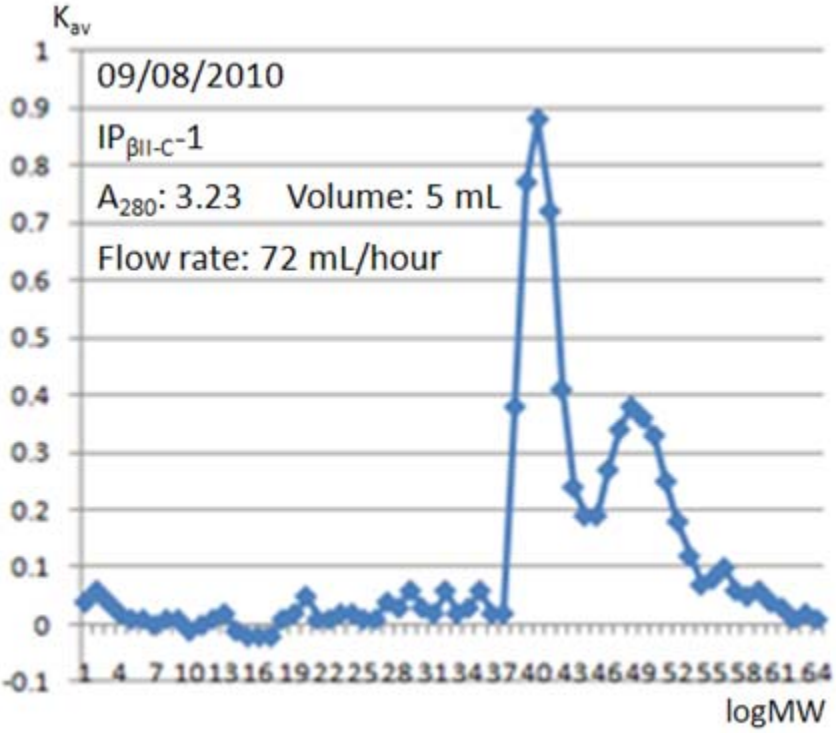

K_{av}
09/08/2010
$IP_{\beta II\text{-}C}$-1
A_{280}: 3.23 Volume: 5 mL
Flow rate: 72 mL/hour
logMW
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 55 58 61 64

Figure 19. Solution property analysis using size-exclusion chromatography of the GST-$IP_{\beta II-C}$-1 fusion protein. (A) The column was calibrated using Ribonuclease A (log MW: 4.1367, K_{av}: 0.4169), Carbonic Anhydrase(log MW: 4.46, K_{av}: 0.2732), Ovalbumin (log MW: 4.644, K_{av}: 0.1592) and Conalbumin (log MW: 4.875, K_{av}: 0.1150) with a flow rate of 72 mL/hour . (B) 5 mL sample with A_{280} of 3.23 was passed through Sephacryl(R) 10-HR column, and the absorbance readings of the corresponding fractions were recorded. The elution volumes (V_E) corresponding to the peaks were 82.5 mL for the first peak (fraction number 28), and 100.5 mL for the second peak (fraction number 34). Based on this calibration, elution profile of GST-$IP_{\beta II-C}$-1 fusion protein sample shows that the solution is composed of 2 main components, first one at 89,949 Da (K_{av}= 0.0695) and second one at 40,654 Da (K_{av}= 0.2133).

D. Discussion

We prepared the GST fusion protein for $IP_{\beta II-C}$-1 that was identified in our screening of human brain cDNA library, using a C-terminal fragment (residues 1697-2145) of βII spectrin, including the tetramerization region, as the bait protein. This protein abolishes the interaction of βII-C fragment with N-terminal region 359 amino acid residues of αII spectrin (αII-N1), which also includes the tetramerization region on αII.

DNA Sequence analysis of the bacterial expression plasmid, pGEX-$IP_{\beta II-C}$-1 showed that the sequence for $IP_{\beta II-C}$-1 was present in its entirety, which was preceded by a complete sequence for the GST tag. Preparation of the GST-$IP_{\beta II-C}$-1 started with the expression of the fusion protein in BL21 codon+ cells. The optimum conditions for the expression of the $IP_{\beta II-C}$-1 fusion protein was determined to be 3 hours of growth at 37 °C in Terrific Broth, and 3 hours of growth at 30 °C after addition of IPTG. Mass analysis of the prepared fusion protein showed that the molecular mass of the GST-$IP_{\beta II-C}$-1 fusion protein sample was 44,062.5 Da, and the expected mass from the sequence was 44,063.0 Da. Large-scale preparation of the GST-$IP_{\beta II-C}$-1 protein produced approximately 1.5 mg of fusion protein per one gram of cells. This was less than the 5 mg of pure protein for other proteins, such as spectrin isoforms purified in our laboratory.

We have documented that GST-$IP_{\beta II-C}$-1 construct has the full GST sequence, therefore, this construct, in theory, might yield similar amounts of proteins, 3 times more than obtained.

Based on the results we obtained, we have successfully produced the library protein with the GST tag. This protein corresponds to a fragment of human brain protein syntaxin binding protein 1 (residues 284 – 428). This protein may further be analyzed by biophysical methods for its interaction with βII-C protein, and more importantly, its impact on the interaction of αII-N and βII-C proteins may be elucidated. This would provide information on the possible regulation of this interaction by this protein.

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CHAPTER 5

CONCLUSION

We have performed two different but inter-related projects involving the non-erythroid spectrin subunit association to form tetramers.

In the first part, we used the Y2H systems to study protein-protein interactions. αII spectrin with mutations V22F, V22M or V22W interacted with βII spectrin at the tetramerization site (N-terminal region of αII and C-terminal region of βII). However, αII-N-V22D did not. Although ITC methods provide protein-protein interaction information quantitatively, ITC methods require not only the preparation of recombinant proteins but also characterization of these proteins for proper functional analysis. Y2H method is relatively simple and is able to qualitatively demonstrate protein-protein interactions.

The αII mutants and β spectrin exhibit different affinities to give different levels of tetramers. As discussed previously (Li and Fung, 2009), αII spectrin has recently been reported to be essential for stabilizing nascent sodium channel clusters (Voas *et al.*, 2007), assembling the mature node of Ranvier (Voas *et al.*, 2007), and regulating endothelial cell-cell contacts (Benz *et al.*, 2008). The tetramer formation of αII-βII spectrin is also essential in the regulatory step for neuritogenesis (Bignone *et al.*, 2007). Tetramerization is clearly important for spectrin function. Some mutations in αII spectrin at the tetramerization region will result in abnormal functions.

In the second part, we identified 17 proteins that interact with the C-terminal region (residues 1697-2145) of βII spectrin. 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, except for the last 8 residues, is identical to a fragment (residues 284-428) of syntaxin binding protein 1. This protein may inhibit or regulate non-erythroid spectrin tetramerization.

These two thesis projects show that either mutation(s) or the presence of interacting proteins will affect the tetramer formation in spectrin leading to abnormal cellular functions that involve spectrin network.

APPENDIX

I - Primers Used

Primers used in our studies are tabulated in Table VI. The general criteria used for the design of the primers were that; they were 25 – 45 base pairs long, did not have complementary sequences at either end, and had a T_m value over 73 °C. The primers listed in this table has been used in different experiments belonging to different studies mentioned, and therefore the aim for the design of the primers are introduced in the first column. Introduced mutations are written in bold, and the restriction enzyme recognition sites (introduced) are underlined.

First two pairs of primers (1 - 4) were used to replace the Val-22 codon (GTC) with others to generate sequences coding for αII-N1-V22W and -V22M proteins, as explained in Chapter 2. First pair (1 and 2) replaces the codon coding for valine with the tryptophane codon (TGG) and the second pair with the methionine codon (ATG).

Primers 5 - 20 were used for our studies explained in Chapter 3. Primers 5 and 6 replaces the present *Bam*HI (GGATCC) and *Eco*RI (GAATTC) sites at the upstream and downstream of the sequence coding for βII-C, with *Eco*RI and *Bam*HI sites, respectively, while amplifying the sequence fragment in order to transfer it from bacterial expression vector, pGEX, to the yeast cloning vector, pBD.

Primers 7 and 8 introduces the same restriction enzyme recognition sites to αII-N sequence in order to transfer it from pGEX to the first cloning site (MCS1) of the yeast three-hybrid vector pBridge. Primers 9 and 10 replaces the present *Bam*HI and *Eco*RI recognition sites of the αII-N sequence with *Not*I (GCGGCCGC) and *Bgl*II (AGATCT) recognition sites, respectively, in order

to transfer it from bacterial expression vector, pGEX, to the second cloning site (MS2) of the pBridge vector.

Primers 11 and 12 introduce *Eco*RI and *Bam*HI sites to the βII-C sequence during its amplification for cloning into the first cloning site of pBridge vector. Due to the presence of internal *Not*I and *Bgl*II recognition sites in the βII-C sequence, these sites were silenced, by replacing the nucleotides without altering the amino acid sequence. For this, primers 13 and 14 were used to silence *Not*I recognition site, ad 15 and 16 were used to silence *Bgl*II site. Successful silencing of the restriction enzyme recognition was verified by the absence restriction enzyme digestion and sequencing. βII-C sequence (without the *Not*I and *Bgl*II recognition sites) was transferred into the second cloning site of the pBridge plasmid using primers 17 and 18, introducing *Not*I and *Bgl*II sites to each ends of the sequence.

Primers 19 and 20 were used to transfer selected library sequences from library plasmids to the second cloning site of the pBridge plasmid, introducing *Not*I and *Bgl*II sites to each ends of the sequences.

The last primer pair was used for studies explained Chapter 4. They were used to transfer the sequence coding for $IP_{\beta II\text{-}C}$-1 sequence from library plasmid, pAD, to bacterial expression plasmid, pGEX-2T.

TABLE VI. Primers used in our studies.

1	Introduce V22W mutation	Forward	5’-GAGAGGCGGCAGCAG**TGG**CT**C**GA**T**CGATACCACCGCTTC-3’
2		Reverse	5'-GAAGCGGTGGTATCGATCGAGCCACTGCTGCCGCCTCTC-3'
3	Introduce V22M mutation	Forward	5’-GAGAGGCGGCAGCAG**AT**G**G**CTAGACCG**G**TACCACCGCTTC-3’
4		Reverse	5'-GAAGCGGTGGTACCGGTCTAGCATCTGCTGCCGCCTCTC-3'
5	Transfer βII-C from pGEX to pBD	Forward	5’GTGAATTCATGGAGCAATTTCCAAAGGAAACCGTTGTG-3’
6		Reverse	5’CGATG**G**AT**C**CTCAATCATTGAGCCGTGCATGTCTCTC-3’
7	Transfer αII-N from pGEX to pBR (MCS1)	Forward	5’-GTG**A**AT**T**CATGGGCCCAAGTGGGGTCAAAGTGCTGG-3’
8		Reverse	5’CGATG**G**AT**C**CTCAATCATTGAGCCGTGCATGTCTCTC-3’
9	Transfer αII-N from pGEX to pBR (MCS2)	Forward	5’-CCG**GCGGCCGC**CATGGACCCAAGTGGGGTCAAAGTGCTG-3’
10		Reverse	5’-CGAT**AG**AT**CT**TCAATCATTGAGCCGTGCATGTCTCTCTGCCGC-3’
11	Transfer βII-C from pGEX to pBR (MS1)	Forward	5’-CTGGTTCCGCGTG**A**AT**T**CAGACACAGGTTATTCCAGCTC-3’
12		Reverse	5’-CAGTCAGTCACGATG**G**AT**C**CTCACACCGTTTCTGCCATC-3’
13	Silence *Not*I Site in βII-C	Forward	5’-CACGATGCCAAGGAGAT**T**TTTGGGCGTATACAGGAC-3’
14		Reverse	5’-GTCCTGTATACGCCC**A**AAAATCTCCTTGGCATCGTG-3’
15	Silence *Bgl*II Site in βII-C	Forward	5’-GAAGAGGAGAGGAAGAG**A**CG**A**CCGCCTTCTCCCGAG-3’
16		Reverse	5’-CTCGGGAGAAGGCGG**T**CG**T**CTCTTCCTCTCCTCTTC-3’
17	Transfer βII-C from pGEX to pBR (MS2)	Forward	5’-TCGGATCTGGTTCCG**GCGGCCGC**CAGACACAGGTTATTC-3’
18		Reverse	5’-CGTCAGTCAGTCACGAT**AG**AT**CT**TCACACCGTTTCTGCC-3’
19	Transfer selected library sequences to pBR (MCS2)	Forward	5’-CCAAGCAGTGGTATCAACGC**GGCCGC**GCCATTATGGCC3’
20		Reverse	5’-CATGTCGGCCGCCTCG**AGA**TCTAGAGG-3’
21	Transfer $IP_{βII-C}$-1 fusion protein sequence from pAD to pGEX	Forward	5’-GAGTGGCCATTA**GGATCC**GGGGACGACGACCTGTGG-3’
22		Reverse	5'-CCAGCGGGAGTT**GAA**TTCTCACCTGTCAGGGCAC-3'

II - DNA and Protein Sequences of $IP_{\beta II\text{-}C}$ s

Sequences of the identified library plasmids are presented below. Sequence analysis results (from RRC) were aligned, and a complete sequence of the plasmid was formed using the common region in the forward and the reverse complement of the reverse sequencing. The common sequence is underlined in the sequences. SMARTIII and CDSIII sequences were identified in the combined sequence, and cDNA sequence was identified as the sequence between these (also excluding the polyA sequence). SMARTIII and CDSIII sequences are written in bold characters and the first and last (stop) codons of the expressed sequences are indicated with a box. Total number of nucleotides in the cDNA sequence is given in parenthesis and the polyA sequence is written in italics.

Finally, the amino acid sequences of $IP_{\beta II\text{-}C}$ s, as determined using the Translate tool of ExPASy Proteomics Server, are presented.

$IP_{\beta II-C}$-1

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGGG**

GACGACGACCTGTGGATAGCACTGCGCCACAAGCACATCGCAGAGGTGTCCCAGGAAGTCACCCGGTCTC
TGAAAGATTTTTCTTCTAGCAAGAGAATGAATACTGGAGAGAAGACCACCATGCGGGACCTGTCCCAGAT
GCTGAAGAAGATGCCTCAGTACCAGAAAGAGCTCAGCAAGTACTCCACCCACCTGCACCTTGCTGAGGAC
TGTATGAAGCATTACCAAGGCACCGTAGACAAACTCTGCCGAGTGGAGCAGGACCTGGCCATGGGCACAG
ATGCTGAGGGAGAGAAGATCAAGGACCCTATGCGAGCCATCGTCCCCATTCTGCTGGATGCCAATGTCAG
CACTTATGACAAAATCCGCATCATCCTTCTCTACATCTTTTTGAAGAATGGCATCACGGAGGAAAACCTG
AACAAACTGATCCAGTCTAAGTCACAGTGCCCTGACAGGTGAGAAGCAAACTCCCGCTGGAAGCCTCCAT
CTCTTTGGAAAAACAGTTAGTCTGGAGCCTGTGGCCCAGGCCCTTCTGTCCCCAGGCATCATCCCAACAG
CTCATTTTCCCTAGTCCGCCTTCGTTCAAGGGTCAGGAATGGACCAGAACAGATGGGTTCTGGAGGCCCC
TGAACAGAGGGCTATGGCTGTGGAGAAGGTTCTTGGCCCGTTGGACTCACACAGACCCTGTACCCTCTCG
GCAAGCATCTTCAGTCAGATTATCCTCAGTTTCAGATACTTCATAATACCTTGTGTTGTGTGGGGTCATA
CATCATCGTGTTTGTAAGAGAAGATGGTCATTTTATTCTCTGTATAAAACTTAGCTCTAAAGCAGAAACT
AAAACAGCAAATGCAGGAAGGCTGTCTCGCCATCCTCAAGACTCAGCAGCTCTCATTCTCCAGTGGTGAG
CACACCATTTGTGCTGCTGCTGTTGTCGTGAAATATAATAACAGTGGAAGTCACAAAAATGTCCCCTGCC
CAGCCCCCTCGCCGCCCTTGACCTCCTGCAGGCCATGTGTGTATTACTTGTCTAGTGATGTCCTCTCAAA
GTGCTGTACGCGAGTTGGGCGCCACCTCCGCCTCCCTTTCAGAGCCTGCTCCCCGCCCTCTTTGCTCGCT
GCATTGTGGTGTTTTTTTCTCAAGGCTTTGAAATCTCCCCTTGCACTGAGATTAGTCGTCAGATCTCTCC
CCGTCTCCCTCCCAACTTATACGCCCTGATTTCCTTAGGACGGAACCGCAGGCACCTGCGCCGGGCGTCT
TACTCCCGCTGCTTGTTCTGTCCCCTCCCTCGGACCAAACAGTGCTCATGTTTCAGGACCTTTTTTGTGG
AAGATGTTGGTTTCCCTTTTTCTTTTATTTATATAAAAATAATTTATCAAAAGGATATTTTAAAAAAGCT
AGTCTGTCTTGAAACTTGTTTACCTTAAAATTATCAGAATCTCAGTGTTTGAAAGTACTGAAGCACAAAC
ATATATCATTTCTGTACCATTCTGTACTAAAGCACTTGAGTTTAATAAATAAAGAAATCAAC (1536)

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-1 amino acid sequence

DDDLWIALRHKHIAEVSQEVTRSLKDFSSSKRMNTGEKTTMRDLSQMLKKMPQYQKELSKYSTHLHLAED
CMKHYQGTVDKLCRVEQDLAMGTDAEGEKIKDPMRAIVPILLDANVSTYDKIRIILLYIFLKNGITEENL
NKLIQSKSQCPDR

$IP_{\beta II-C}$-2

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCTG**

AGTTCTTCCTTTGACAAGATGGCGGCAGGAGGCAGTGGCGTTGGTGGGAAGCGCAGCTCGAAAAGCGATG
CCGATTCTGGTTTCCTGGGGCTGCGGCCCACTTCGGTGGACCCAGCGCTGAGGCGGCGGCGGCGAGGCCC
AAGAAATAAGAAGCGGGGCTGGCGGCGGCTTGCTCAGGAGCCGCTGGGGCTGGAGGTCGACCAGTTCCTG
GAAGACGTGCGGCTACAGGAGCGCACGAGCGGTGGCTTGTTGTCAGAGGCCCCAAATGAAAAACTCTTCT
TCGTGGACACTGGCTCCAAGGAAAAAGGGCTGACAAAGAAGAGAACCAAAGTCCAGAAGAAGTCACTGCT
TCTCAAGAAACCCCTTCGGGTTGACCTCATCCTCGAGAACACATCCAAAGTCCCTGCCCCCAAAGACGTC
CTCGCCCACCAGGTCCCCAACGCCAAGAAGCTCAGGCGGAAGGAGCAGCTATGGGAGAAGCTGGCCAAGC
AGGGCGAGCTGCCCCGGGAGGTGCGCAGGGCCCAGGCCCGGCTCCTCAACCCTTCTGCAACAAGGGCCAA
GCCCGGGCCCCAGGACACCGTAGAGCGGCCCTTCTACGACCTCTGGGCCTCAGACAACCCCCTGGACAGG
CCGTTGGTTGGCCAGGATGAGTTTTTCCTGGAGCAGACCAAGAAGAAAGGAGTGAAGCGGCCAGCACGCC
TGCACACCAAGCCGTCCCAGGCACCCGCCGTGGAGGTGGCGCCTGCCGGAGCTTCCTACAATCCATCCTT
TGAAGACCACCAGACCCTGCTCTCAGCGGCCCACGAGGTGGAGTTGCAGCGGCAGAAGGAGGCGGAGAAG
CTGGAGCGGCAGCTGGCCCTGCCCGCCACGGAGCAGGCCGCCACCCAGGAGTCCACATTCCAGGAGCTGT
GCGAGGGGCTGCTGGAGGAGTCGGATGGTGAGGGGGAGCCAGGCCAGGGCGAGGGGCCGGAGGCTGGGGA
TGCCGAGGTCTGTCCCACGCCCGCCCGCCTGGCCACCACAGAGAAGAAGACGGAGCAGCAGCGGCGGCGG
GAGAAGGCTGTGCACAGGCTGCGGGTACAGCAGGCCGCGTTGCGGGCCGCCCGGCTCCGGCACCAGGAGC
TGTTCCGGCTGCGCGGGATCAAGGCCCAGGTGGCCCTGAGGCTGGCGGAGCTGGCGCGGCGGCAGAGGCG
GCGGCAGGCGCGGCGGGAGGCTGAGGCTGACAAGCCCCGAAGGCTGGGGCGGCTCAAGTACCAGGCACCT
GACATCGACGTGCAGCTGAGCTCGGAGCTGACAGACTCGCTCAGGACCCTGAAGCCCGAGGGCAACATCC
TTCGAGACCGGTTCAAGAGCTTCCAGAGGAGGAATATGATCGAGCCTCGAGAGAGAGCCAAGTTCAAACG
CAAGTACAAGGTGAAGCTGGTGGAGAAGCGGGCGTTCCGTGAGATCCAGTTGTAGCTGCCATCAGATGCC
GGAGACTCGCCCTTCAATAAAAAATCTTTTTTAGCTT (1509)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-2 amino acid sequence

SSFDKMAAGGSGVGGKRSSKSDADSGFLGLRPTSVDPALRRRRRGPRNKKRGWRRLAQEPLGLEVDQFLE
DVRLQERTSGGLLSEAPNEKLFFVDTGSKEKGLTKKRTKVQKKSLLLKKPLRVDLILENTSKVPAPKDVL
AHQVPNAKKLRRKEQLWEKLAKQGELPREVRRAQARLLNPSATRAKPGPQDTVERPFYDLWASDNPLDRP
LVGQDEFFLEQTKKKGVKRPARLHTKPSQAPAVEVAPAGASYNPSFEDHQTLLSAAHEVELQRQKEAEKL
ERQLALPATEQAATQESTFQELCEGLLEESDGEGEPGQGEGPEAGDAEVCPTPARLATTEKKTEQQRRRE
KAVHRLRVQQAALRAARLRHQELFRLRGIKAQVALRLAELARRQRRRQARREAEADKPRRLGRLKYQAPD
IDVQLSSELTDSLRTLKPEGNILRDRFKSFQRRNMIEPRERAKFKRKYKVKLVEKRAFREIQL

$IP_{\beta II-C}$-3

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCTG**

AGTTCTTCCTTTGACAAGATGGCGGCAGGAGGCAGTGGCGTTGGTGGGAAGCGCAGCTCGAAAAGCGATG
CCGATTCTGGTTTCCTGGGGCTGCGGCCCACTTCGGTGGACCCAGCGCTGAGGCGGCGGCGGCGAGGCCC
AAGAAATAAGAAGCGGGGCTGGCGGCGGCTTGCTCAGGAGCCGCTGGGGCTGGAGGTCGACCAGTTCCTG
GAAGACGTGCGGCTACAGGAGCGCACGAGCGGTGGCTTGTTGTCAGAGGCCCCAAATGAAAAACTCTTCT
TCGTGGACACTGGCTCCAAGGAAAAAGGGCTGACAAAGAAGAGAACCAAAGTCCAGAAGAAGTCACTGCT
TCTCAAGAAACCCCTTCGGGTTGACCTCATCCTCGAGAACACATCCAAAGTCCCTGCCCCCAAAGACGTC
CTCGCCCACCAGGTCCCCAACGCCAAGAAGCTCAGGCGGAAGGAGCAGCTATGGGAGAAGCTGGCCAAGC
AGGGCGAGCTGCCCCGGGAGGTGCGCAGGGCCCAGGCCCGGCTCCTCAACCCTTCTGCAACAAGGGCCAA
GCCCGGGCCCCAGGACACCGTAGAGCGGCCCTTCTACGACCTCTGGGCCTCAGACAACCCCCTGGACAGG
CCGTTGGTTGGCCAGGATGAGTTTTTCCTGGAGCAGACCAAGAAGAAAGGAGTGAAGCGGCCAGCACGCC
TGCACACCAAGCCGTCCCAGGCACCCGCCGTGGAGGTGGCGCCTGCCGGAGCTTCCTACAATCCATCCTT
TGAAGACCACCAGACCTGCTCTCAGCGGCCCACGAGGTGGAGTTGCAGCGGCAGAAGGAGGCGGAGAAGC
TGGAGCGGCAGCTGGCCCCTGCCCGCCACGGAGCAGGCCGCCACCCAGGAGTCCACATTCCAGGAGCTGT
GCGAGGGGCTGCTGGAGGAGTCGGATGGTGAGGGGGAGCCAGGCCAGGGCGAGGGGCCGGAGGCTGGGGA
TGCCGAGGTCTGTCCCACGCCCGCCCGCCTGGCCACCACAGAGAAGAAGACGGAGCAGCAGCGGCGGCGG
GAGAAGGCTGTGCACAGGCTGCGGGTACAGCAGGCCGCGTTGCGGGCCGCCCGGCTCCGGCACCAGGAGC
TGTTCCGGCTGCGCGGGATCAAGGCCCAGGTGGCCCTGAGGCTGGCGGAGCTGGCGCGGCGGCAGAGGCG
GCGGCAGGCGCGGCGGGAGGCTGAGGCTGACAAGCCCCGAAGGCTGGGGCGGCTCAAGTACCAGGCACCT
GACATCGACGTGCAGCTGAGCTCGGAGCTGACAGACTCGCTCAGGACCCTGAAGCCCGAGGGCAACATCC
TTCGAGACCGGTTCAAGAGCTTCCAGAGGAGGAATATGATCGAGCCTCGAGAGAGAGCCAAGTTCAAACG
CAAGTACAAGGTGAAGCTGGTGGAGAAGCGGGCGTTCCGTGAGATCCAGTTGTAGCTGCCATCAGATGCC
GGAGACTCGCCCTTCAATAAAAAATTTTTTTTAGCTT (1509)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-3 amino acid sequence

SSFDKMAAGGSGVGGKRSSKSDADSGFLGLRPTSVDPALRRRRRGPRNKKRGWRRLAQEPLGLEVDQFLE
DVRLQERTSGGLLSEAPNEKLFFVDTGSKEKGLTKKRTKVQKKSLLLKKPLRVDLILENTSKVPAPKDVL
AHQVPNAKKLRRKEQLWEKLAKQGELPREVRRAQARLLNPSATRAKPGPQDTVERPFYDLWASDNPLDRP
LVGQDEFFLEQTKKKGVKRPARLHTKPSQAPAVEVAPAGASYNPSFEDHQTCSQRPTRWSCSGRRRRRSW
SGSWPLPATEQAATQESTFQELCEGLLEESDGEGEPGQGEGPEAGDAEVCPTPARLATTEKKTEQQRRRE
KAVHRLRVQQAALRAARLRHQELFRLRGIKAQVALRLAELARRQRRRQARREAEADKPRRLGRLKYQAPD
IDVQLSSELTDSLRTLKPEGNILRDRFKSFQRRNMIEPRERAKFKRKYKVKLVEKRAFREIQL

$IP_{\beta II-C}$-4

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGGG**

CGCGTGGGAAATTCTGCCGGGCAGAGTTTGAACAAACCCAATATTCACAAGAGAGTTTTAACAGAAGCTA
CCGTGGGCAGGGAAAGATCTTTGGGAGAAAGAACCCAAGAGTGTAGTGCATTTGATAGAAACTTGAATCT
GGACCAAAATGTTGTTAGACTTCAAAGAAATAAAACAGGAGAGAGGGTCTTTAAATGTGATATATGCAGC
AAAACCTTCAAATATAATTCAGACCTAAGTAGACACCAGAGAAGTCACACTGGGGAGAAGCCGTACGAAT
GTGGCCGGTGTGGGCGAGCCTTTACTCACAGCTCAAATCTTGTTCTGCACCATCACATTCACACTGGAAA
TAAACCATTTAAATGTGATGAATGTGGGAAAACTTTTGGACTCAATTCTCACCTCCGTCTTCATCGGAGA
ATTCACACTGGAGAAAAACCCTTTGGCTGTGGTGAGTGTGGGAAGGCTTTCAGTCGAAGCTCAACTCTTA
TTCAACATCGGATCATTCACACAGGAGAGAAACCCTACAAGTGTAATGAATGTGGAAGAGGCTTTAGCCA
GAGCCCCCAGTTAACTCAGCATCAGAGAATTCACACTGGAGAGAAGCCGCATGAATGCAGTCACTGTGGG
AAGGCCTTCAGTCGAAGCTCCAGCCTTATTCAGCATGAGAGAATTCACACTGGAGAGAAGCCCCATAAAT
GCAATCAGTGTGGGAAGGCCTTCAGTCAGAGCTCAAGCCTTTTCCTCCATCATCGGGTTCATACTGGAGA
GAAACCCTATGTATGTAATGAATGCGGCAGAGCCTTTGGTTTTACTCTCATCTTACCGAACACGTAAGGA
TTCACACAGGAGAAAAACCCTATGTTTGTTAATGAGTGCGGCAAAGCCTTTCGTCGGAGTGCACTCTTGT
TCAGCATCGAAGAGTTCACACTGGGGAGAAGCCCTACCAGTGCGTTGAATGTGGGAAAGCTTTCAGCCAG
AGCTCCCAGCTCACCCTACATCAGCGAGTTCACACTGGAGAGAAGCCCTATGACTGTGGTGACTGTGGGA
AGGCCTTCAGCCGGAGGTCAACCCTCATTCAGCATCAGAAAGTTCACAGCGGAGAGACTCGTAAGTGCAG
AAAACATGGTCCAGCCTTTGTTCATGGCTCCAGCCTCACAGCAGATGGACAGACTCCCACTGGAGAGAAG
CACGGCAGAGCCTTTAACCATGGTGCAAATCTCATTTTGCGCTGGACAGTTCACACTGGTGAGAAATCCT
TTGGATGTAATGAATATGGAAAAGCTTTCAGTCCCACCTCCCGACCCACTGAAGATCAGATAATGCATGC
TGGGGAAAAGCCCTATAAATGTCAAGAATGTGGAAACGCCTTCAGTGGAAAGTCAACCCTTATTCAACAT
CAGGTAACTCACACTGGTCAGAAACCATGTCATTGCAGTGTGTATGGGAAAGCCTTCAGCCAGAGTTCAC
AGCTCACACCACCTCAGCAGACTCGTGTTGGAGAGAAACCTGCTTTAAATGATGGCTTTAAAAGATACTT
TATTCATATCAAGAAGATTTTCCAAGAAAGACATTTTTAATGTGATAAATGCAGAAGACAGTTTAGCAAC
TGTTCACTTGACATTAGAAGATAAGATGGCATAATGAAAGATATATAAGGTTTAAATATTACTGGCAAAG
TAAAATAAATAGTTCAGATGACTACTAAAGTCAAAGTCATTAAATTTGGAAGTAAACAAG (1744)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-4 amino acid sequence

RVGNSAGQSLNKPNIHKRVLTEATVGRERSLGERTQECSAFDRNLNLDQNVVRLQRNKTGERVFKCDICS
KTFKYNSDLSRHQRSHTGEKPYECGRCGRAFTHSSNLVLHHHIHTGNKPFKCDECGKTFGLNSHLRLHRR
IHTGEKPFGCGECGKAFSRSSTLIQHRIIHTGEKPYKCNECGRGFSQSPQLTQHQRIHTGEKPHECSHCG
KAFSRSSSLIQHERIHTGEKPHKCNQCGKAFSQSSSLFLHHRVHTGEKPYVCNECGRAFGFTLILPNT

$IP_{\beta II-C}$-5

Reliable sequence

AAGAAGGGATCTTTAATACGACTCACTATAGGGCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGA
TTACGCTCATATGGCCATGGAGGCCAGTGAATTCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTA**
TGGCCGGGG

CGCGTGGGAAATTCTGCCGGGCAGAGTTTGAACAAACCCAATATTCACAAGAGAGTTTTAACAGAAGCTA
CCGTGGGCAGGGAAAGATCTTTGGGAGAAAGAACCCAAGAGTGTAGTGCATTTGATAGAAACTTGAATCT
GGACCAAAATGTTGTTAGACTTCAAAGAAATAAAACAGGAGAGAGGGTCTTTAAATGTGATATATGCAGC
AAAACCTTCAAATATAATTCAGACCTAAGTAGACACCAGAGAAGTCACACTGGGGAGAAGCCGTACGAAT
GTGGCCGGTGTGGGCGAGCCTTTACTCACAGCTCAAATCTTGTTCTGCACCATCACATTCACACTGGAAA
TAAACCATTTAAATGTGATGAATGTGGGAAAACTTTTGGACTCAATTCTCACCTCCGTCTTCATCGGAGA
ATTCACACTGGAGAAAAACCCTTTGGCTGTGGTGAGTGTGGGAAGGCTTTCAGTCGAAGCTCAACTCTTA
TTCAACATCGGATCATTCACACAGGAGAGAAACCCTACAAGTGTAATGAATGTGGAAGAGGCTTTAGCCA
GAGCCCCCAGTTAACTCAGCATCAGAGAATTCACACTGGAGAGAAGCCCTATGACTGTGGTGACTGTGGG
AAGGCCTTCAGCCGGAGGTCAACCCTCATTCAGCATCAGAAAGTTCACAGCGGAGAGACTTGTAAGTGCA
GAAAACATGGTCCAGCCTTTGTTCATGGCTCCAGCCTCACAGCAGATGGACAGACTCCCACTGGAGAGAA
GCACGGCAGAGCCTTTAACCATGGTGCAAATTTCATTTTGCGCTGGACAGTTCACACTGGTGAGAAATCC
TTTGGATGTAATGAATATGGAAAAGCTTTCAGTCCCCCCTCCCGACCCCCTGAAGATCAGATAATGCATG
CTGGGGAAAAGCCCTATAAATGTCAAGAATGTGGAAACGCCTTCAGTGGAAAGTCAACCCTTATTCAACA
TCAGGTAACTCACACTGGTCAGAAACCATGTCATTGCAGTGTGTATGGGAAAGCCTTCAGCCAGAGTTCA
CAGCTCACCCCCCCTCAGCAGACTCGTGTTGGAGAGAAACCTGCTTTAAATGATGGCTTTAAAAGATACT
TTATTCATATCAAGAAGATTTTCCAAGAAAGACATTTTTAATGTGATAAATGCAGAAGACAGTTTAGCAA
CTGTTCACTTGACATTAGAAGATAAGATGGCATAATGAAAGATATATAAGGTTTAAATATTACTGGCAAA
GTAAAATAAATAGTTCAGATGACTACTAAAGTCAAAGTCATTAAATTTGGAAGTAAACAAG (1325)

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CATGTCGGCCGCCTCGGCCTCTAGAGGGTGGGCATCGATACGGGACCATCGAGCTCGAGCTGCAAGATGG
T

$IP_{\beta II-C}$-5 amino acid sequence

RVGNSAGQSLNKPNIHKRVLTEATVGRERSLGERTQECSAFDRNLNLDQNVVRLQRNKTGERVFKCDICS
KTFKYNSDLSRHQRSHTGEKPYECGRCGRAFTHSSNLVLHHHIHTGNKPFKCDECGKTFGLNSHLRLHRR
IHTGEKPFGCGECGKAFSRSSTLIQHRIIHTGEKPYKCNECGRGFSQSPQLTQHQRIHTGEKPYDCGDCG
KAFSRRSTLIQHQKVHSGETCKCRKHGPAFVHGSSLTADGQTPTGEKHGRAFNHGANFILRWTVHTGEKS
FGCNEYGKAFSPPSRPPEDQIMHAGEKPYKCQECGNAFSGKSTLIQHQVTHTGQKPCHCSVYGKAFSQSS
QLTPPQQTRVGEKPALNDGFKRYFIHIKKIFQERHF

$IP_{\beta II-C}$-6

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGGGG**

AAAAAGAAAATCAATGGCAGTAGCCCTGACACAGCCACTTCTGGTGGTTACCACTCACCTGGGGATTCAG
CAACAGGTATCTACGGGGAGGGCCGTGCATCCTCTACTACCCTGGAGGATCTGGAGAGCCAGTACCAAGA
ACTAGCAGTGGCCCTGGATTCAAGCTCCGCAATAATCAGTCAACTCACTGAAAACATCAATTCACTGGTT
CGCACATCTAAGGAGGAGAAGAAGCATGAGATACATCTGGTACAGAAGCTTGGGAGGAGCTTGTTCAAAC
TCAAAAACCAGACGGCTGAACCCCTGGCCCCAGAGCCCCCAGCAGGGCCATCTAAGGTAGAGCAGCTACA
AGATGAGACCAACCACCTAAGGAAGGAGCTAGAGAGTGTGGGAAGACAGCTCCAGGCTGAGGTGGAAAAC
AATCAGATGTTGAGTCTCCTGAACAGGAGACAGGAGGAGAGGCTACGTGAACAGGAGGAGAGGCTACGTG
AACAGGAGGAGAGGCAACGTGAACAGGAGGATAGGCTACATGAACAGGAGGAGAGGCTATGTGAACAGGA
GGAGAGGCTACGTGAACAGGAGGAGAGGCTGTGTGAACAGGAGAAGCTGCCAGGGCAGGAGAGGCTGCTG
GAAGAGGTGGAGAAGCTGTTAGAACAGGAGAGGCGGCAGGAGGAGCAGGAGAGGCTGCTGGAGAGGGAGA
GGCTGCTGGAAGAGGTGGAGAAGCTGTTAGAACAGGAGAGGCAGCAGGAGGAGCAGGAGAGGCTGCTGGA
GAGGGAGAGGCTGCTGGAAGAGGTGGAGAAGCTGTTAGAACAGGAGAGGCGGCAGGAGGAGCAGGAGAGG
CTGCTGGAGAGGGAGAGGCTGCTGGACGAGGTGGAGGAGCTCCTGGACGAGGTGGAGGAGCTCCTGGAGT
AGGAGAGGCTTTGGCAACAGGATGAGAGGCTGTGGCAGCAGTAGACTTTGCAGGAGCTGGAGAGGCTGCG
GGAGCTGGAGAGGCTGCGGGAGCTGGAGAGGATGCTGGAGCTGGGGTGGGAAGCCCTGTACGAGCAGCGG
GCCGAGCCACGCAGCGGCTTTGAGGAGCTGAACAACGAGAACAAGAGCACACTGCAGTTGGAGCAGCAAG
TAAAGGAGCTGAAGAAGTCGGGTGGAGCTGAAGAGCCAAGAGGCTCCGAGTTTGCAGCAGCAGCCAGACC
AGTAGCTGGAGCCCCAGTCCCCCAAGGAGCTTGGATGTGCGGACAAGCAGGGTGGACTCCCCAGGAGCAC
CCAGGCTTGAGTGGGGAAGCTGTTGGTACAGGAGAGGCGGCAGGAGGAGCAGGAGAGGCTGCATGCCATT
TTTTTCGGGCTGCGGAGAACAGGGAGGTAAACATCACCATCATTTAAGAGCGGGTCAAGGAATTG
(1400)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-6 amino acid sequence

KKKINGSSPDTATSGGYHSPGDSATGIYGEGRASSTTLEDLESQYQELAVALDSSSAIISQLTENINSLV
RTSKEEKKHEIHLVQKLGRSLFKLKNQTAEPLAPEPPAGPSKVEQLQDETNHLRKELESVGRQLQAEVEN
NQMLSLLNRRQEERLREQEERLREQEERQREQEDRLHEQEERLCEQEERLREQEERLCEQEKLPGQERLL
EEVEKLLEQERRQEEQERLLERERLLEEVEKLLEQERQQEEQERLLERERLLEEVEKLLEQERRQEEQER
LLERERLLDEVEELLDEVEELLE

$IP_{\beta II-C}$-7

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG**

GGGGGGCAGCCAGGCCTGGCTCGAGATGCCGAAGTCGTGCGCGGCCCGGCAGTGCTGCAACCGCTACAGC
AGCCGCAGGAAGCAGCTCACCTTCCACCGGTTTCCGTTCAGCCGCCCGGAGCTGCTGAAGGAATGGGTGC
TGAACATCGGCCGGGGCAACTTCAAGCCCAAGCAGCACACGGTCATCTGCTCCGAGCACTTCCGGCCAGA
GTGCTTCAGCGCCTTTGGAAACCGCAAGAACCTAAAGCACAATGCCGTGCCCACGGTGTTCGCCTTTCAG
GACCCCACACAGCAGGTGAGGGAGAACACAGACCCTGCCAGTGAGAGAGGAAATGCCAGCTCTTCTCAGA
AAGAAAAGGTCCTCCCTGAGGCGGGGGCCGGAGAGGACAGTCCTGGGAGAAACATGGACACTGCACTTGA
AGAGCTTCAGTTGCCCCCAAATGCCGAAGGCCACGTAAAACAGGTCTCGCCACGGAGGCCGCAAGCAACA
GAGGCTGTTGGCCGGCCGACTGGCCCTGCAGGCCTGAGAAGGACCCCCAACAAGCAGCCATCTGATCACA
GCTATGCCCTTTTGGACTTAGATTCCCTGAAGAAAAAACTCTTCCTCACTCTGAAGGAAAATGAAAAGCT
CCGGAAGCGCTTGCAGGCCCAGAGGCTGGTGATGCGAAGGATGTCCAGCCGCCTCCGTGCTTGCAAAGGG
CACCAGGGACTCCAGGCCAGACTTGGGCCAGAGCAGCAGAGCTGAGCCCCACAGGCTCCGGACGCAGAGG
TGGCAGTGGCACCAGGGCCGGCAGAGCTTTGGAGCTCTGGCTGTGGACATTTTTGTTTGCTGTGGACACT
GAGAAAGTTGGCCATGAGGCCTGCTTGGCCGGGGATCGAGACAGTAGCCAAGCTCCCCGGCGAGAGCCCC
AATGCCGTTTGGGGGACGTTTAGAGGCGTGGCACTAGGAGTGCACATCTGTGAGCATGACAAGCTTATCC
TCCCATGGTAACAGAAGTCCAGGCTGAGGCTGATTTTGGACGCTGTCCTTTCAGCACACGCAGAGCAAAG
ATCGTTGGAAGCCCCAGTGTGGGAGATGCTCCTCAGGGAGGAAGCCATGTGAGGGGGCTGGCTCTGTGGC
GGGTGAGTGGTCCCCTCCTCCATCAGCCTGGACAGCCGCTCGGGGTTTTAAGGAGTGACTCCTGTCCCGG
CCTGGTGTGAGTGGGCAGTGTAATAAAGTGTCTTTTTAC (1232)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-7 amino acid sequence

GGSQAWLEMPKSCAARQCCNRYSSRRKQLTFHRFPFSRPELLKEWVLNIGRGNFKPKQHTVICSEHFRPE
CFSAFGNRKNLKHNAVPTVFAFQDPTQQVRENTDPASERGNASSSQKEKVLPEAGAGEDSPGRNMDTALE
ELQLPPNAEGHVKQVSPRRPQATEAVGRPTGPAGLRRTPNKQPSDHSYALLDLDSLKKKLFLTLKENEKL
RKRLQAQRLVMRRMSSRLRACKGHQGLQARLGPEQQS

$IP_{\beta II-C}$-8

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGGGGGGG**

GGGGGAAGGAGCAGCACCAAATCCAAGATGGCGGCCAGCAGGAGGCTGATGAAGGACAACCCTCCATATG
ATAAGGGAGCCTTCAGAATCGAAATCAACTTTCCAGCAGAGTACCCATTCAAACCACCGAAGATCACATT
TAAAACAAAGATCTATCACCCAAACATCGACGAAAAGGGGCAGGTCTGTCTGCCGGTAATTAGTGCCGAA
AACTGGAAGCCAGCAACCAAAACCGACCAAGTAATCCAGTCCCTCATAGCACTGGTGAATGACCCCCAGC
CTGAGCACCCGCTTCGGGCTGACCTAGCTGAAGAATACTCTAAGGACCGTAAAAAATTCTGTAAGAATGC
TGAAGAGTTTACAAAGAAATATGGGGAAAAGCGACCTGTGGACTAAAATCTGCCACGATTGGTTCCAGCA
AGTGTGAGCAGAGACCCCGTGCAGTGCATTCAGACACCCCGCAAAGCAGGACTCTGTGGAAATTGACACG
TGCCACCGCCTGGCGTTCGCTTGTGGCAGTTACTAACTTTCTACAGTTTTCTTAATCAAAAGTGGTCTAG
GTAACCTGTAAAGAAAGGATTAAAAATTTAAGATGTTC (602)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-8 amino acid sequence

GGRSSTKSKMAASRRLMKDNPPYDKGAFRIEINFPAEYPFKPPKITFKTKIYHPNIDEKGQVCLPVISAE
NWKPATKTDQVIQSLIALVNDPQPEHPLRADLAEEYSKDRKKFCKNAEEFTKKYGEKRPVD

$IP_{\beta II-C}$-9

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCA**

GAGGCAGCAGATCCCCTAGCATCTAAACTTAACAAGGTCACCCAATTGACAGGTTTCTCAGATCCTGTAT
ATGCAGAAGCTTACGTTCATGTCAACCAATATGATATTGTCCTGGATGTACTTGTTGTGAACCAAACCAG
TGATACTTTGCAGAATTACACATTAGAACTAGCTACACTAGGGGATCTGAAACTTGTGGAAAAGCCGTCT
CCTTTGACTCTTGCTCCTCATGACTTCGCAAATATTAAAGCTAACGTCAAAGTAGCATCAACAGAAAATG
GAATAATTTTTGGTAATATAGTTTATGATGTCTCTGGAGCAGCAAGTGACAGAAATTGTGTGGTTCTCAG
TGATATTCACATCGACATCATGGACTATATCCAGCCTGCAACTTGCACTGATGCAGAATTCCGTCAGATG
TGGGCCGAATTTGAATGGGAAAACAAAGTGACAGTTAACACCAACATGGTTGATTTAAATGACTACTTAC
AGCACATATTAAAGTCAACCAATATGAAATGCCTGACTCCAGAAAAGGCCCTTTCTGGTTACTGTGGCTT
TATGGCAGCCAACCTTTATGCTCGTTCCATATTTGGTGAAGATGCACTTGCAAATGTCAGCATTGAGAAG
CCAATTCACCAGGGACCAGATGCTGCTGTTACCGGCCATATAAGAATTCGTGCAAAGAGCCAGGGAATGG
CCTTAAGTCTTGGAGATAAAATCAACTTGTCACAGAAGAAAACTAGTATATAAAAATAAACAAAAAGTCC
TTGAAGCTTTACAGTTAATTTAGGTATGGGCTTACTGGACTCCAACATCTTTTGTACTCTTTCATGCTTA
TATAGAATTTGAGTTCATGCTGAATACTTTTCAGCCAATAATTTATAGCCTTTCCCTTAAATCAAGATTG
AGTTTAAAATTATAGTTTGTCTTTTGTTTTAACAGTTTTGAATGCTGTCCTCAAAGTATATAATGTTTCA
TGTACCAAGACCCTTTTCACAGTACAATAAACAGATCTATTCATG (1026)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-9 amino acid sequence

EAADPLASKLNKVTQLTGFSDPVYAEAYVHVNQYDIVLDVLVVNQTSDTLQNYTLELATLGDLKLVEKPS
PLTLAPHDFANIKANVKVASTENGIIFGNIVYDVSGAASDRNCVVLSDIHIDIMDYIQPATCTDAEFRQM
WAEFEWENKVTVNTNMVDLNDYLQHILKSTNMKCLTPEKALSGYCGFMAANLYARSIFGEDALANVSIEK
PIHQGPDAAVTGHIRIRAKSQGMALSLGDKINLSQKKTSI

$IP_{\beta II-C}$-10

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGAATACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCGGG**

GAGCTTGGGAGAGATCAGAACACAGTGGAGACCTTACAGAGAATGCACACTACATTTGGGCATGACATCC
AGGCTCTGGGCACACAGGTGAGGCAGCTGCAGGAGGATGCAGCCCGCCTCCAGGCGGCCTATGCGGGTGA
CAAGGCCGACGATATCCAGAAGCGCGAGAACGAGGTCCTGGAAGCCTGGAAGTCCCTCCTGGACGCCTGT
GAGAGCCGCAGGGTGCGGCTGGTGGACACAGGGGACAAGTTCCGCTTCTTCAGCATGGTGCGCGACCTCA
TGCTCTGGATGGAGGATGTCATCCGGCAGATCGAGGCCCAGGAGAAGCCAAGGGATGTATCATCTGTTGA
ACTCTTAATGAATAATCATCAAGGCATCAAAGCTGAAATTGATGCACGTAATGACAGTTTCACAACCTGC
ATTGAACTTGGGAAATCCCTGTTGGCGAGAAAACACTATGCATCTGAGGAGATCAAGGAAAAATTACTGC
AGTTGACGGAAAAGAGGAAAGAAATGATCGACAAGTGGGAAGACCGATGGGAATGGTTAAGACTGATTCT
GGAGGTCCATCAGTTCTCAAGAGACGCCAGTGTGGCCGAGGCCTGGCTGCTTGGACAGGAGCCGTACCTA
TCCAGCCGAGAGATAGGCCAGAGCGTGGACGAGGTGGAGAAGCTCATCAAGCGCCACGAGGCATTTGAAA
AGTTTGCAGCAACCTGGGATGAGAGGTTTTCTGCCCTGGAAAGGCTGACTACATTGGAGTTACTGGAAGT
GCGCAGACAGC (784)

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CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-10 amino acid sequence

ELGRDQNTVETLQRMHTTFGHDIQALGTQVRQLQEDAARLQAAYAGDKADDIQKRENEVLEAWKSLLDAC
ESRRVRLVDTGDKFRFFSMVRDLMLWMEDVIRQIEAQEKPRDVSSVELLMNNHQGIKAEIDARNDSFTTC
IELGKSLLARKHYASEEIKEKLLQLTEKRKEMIDKWEDRWEWLRLILEVHQFSRDASVAEAWLLGQEPYL
SSREIGQSVDEVEKLIKRHEAFEKFAATWDERFSALERLTTLELLEVRRQ

$IP_{\beta II-C}$-11

Reliable sequence

ACGACTCACTATAGGGCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCA
TGGAGGCCAGTGAATTCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGGGG**

CTTGGAAAGATGGCGTCCCGCAAGGAAGGTACCGGCTCTACTGCCACCTCTTCCAGCTCCACCGCCGGCG
CAGCAGGGAAAGGCAAAGGCAAAGGCGGCTCGGGAGATTCAGCCGTGAAGCAAGTGCAGATAGATGGCCT
TGTGAGTGCTGTTCTTTTTCCTCTGGCCCACGTGGGGAGCGCAGTTCCCAGCCCCGTCCCACCAGACGTA
CTTTCTAGCATTCTCCCCCTTGGGCTTGGTGTGGCTGGGCCAAAGTTTTTCCCTAGTACGTTTCTCTTTC
ATCTTCACTTTTTGTTCAGGAGCTTCGACAGCCTTCTCTTGTCTTCTAACGGGACAGTTGGGGCTTCAGG
AAGGGGACTGAGAATAGGGGTGTGAGTGACAGGTTCCTGGGGTCTTGTTTTGAGATGACCGGTGGGGGGA
TTTGGAGAAAGACTCCTGGAGACCAGCAGCTAACGAAGGTGTCGAGAAGGATTTAGGGACCTAGTAGGGC
TCTGGGAAGGAGAGAGTAAGGAAACTCCACTGATTCCCTGCTGGGATCTGACATCTATAACGTTCCAAGT
GGAGATGTACTTTGTAGCCACTCTTTTGAGCCTGTACTTCGGCGCTTTATCTGAAACCGTATAATTTAGA
ATACTCTTTTGTAGTGTTCATTCTGAAAAACATAAAAGAATATCAAAGAGAAGGGCCTTAAATGTATTTA
GTAGTTAAGGTTCATGAACTAGGACATCCAAGGAAATTCTTGATAAGGAAATGGACTTAGCTGGGAAGAG
GAAAATCTAGAATGAGAAATTTTATTTCTGTTGTTTCGAAGCAAAATGCATTTATATGACGTTCTAGTTT
TAAAAGTATCCTTTCTTGCCCTCTTTTAAAAAGCATCCTCTCTTCCTTTGATTTTTTTAAAGGTTATTTC
CTATTAATGCAGATCTTAGATATTTGTAATAAGAAAGTACTTTTAAATTCATGTGAAAATTGTAATTGCA
AGCAGGTATTTTTAAGTGGAAACTGTTGATAATTTAAACGCAGCATATAATTGAATCTGTTGAACTGACT
TAGTTAAACTGGATACAAGAGTGTAGCCTGGTGGTTAAGAGCTCATTCAGTCTTCTAGGCTCTTCCACTT
AACCAGCTCTGTGACTTTAGGCAAGTTAGCCTTTCCAGGCTTTGCTTTCCTCATCTGTCAAGTGGGAATG
AGAAGTATCTGTATCATAGAGTTGTTTGGATTAAATGAAAAAGTTTATGTGGTGACCTGTAGTCCAGTGT
AATCACTTAATGTTAATTATGATTATTATTATGAGTTTAATGTCTGTGGTTAGCAGAGTTTGAAAGATGC
AGGCAAGAGATTAACTTTGGAATGTTTTGTTTTAAATGTCACTTGTGTAATACTTTGAAAATGCAGGCCG
GGCGGGGTGGCTCACGGCTGTAATCCCAGCACTTTGGGAGGCCGGGGCGGGGGGATCAGGAGGTCAGGAG
TTCGAGACCACCCTGACCAGCATGGTGAAACCCCGTCTTTACTAAAAACACAAAATTAGCCGGGGGGGTT
GGCACGCGCCGGTAGTTTCAGCTACTCGGGAGGCTGAGGCGGGAGAATCGCTTGAACCTGGGGGGGGGGG
GTTGCAGTGAGCCGAGATTGTGCCACTGCACTCCAGCCTGGGCAATAGAGCGAGCCTCCGTCC (1678)

AAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-11 amino acid sequence

LGKMASRKEGTGSTATSSSSTAGAAGKGKGKGGSGDSAVKQVQIDGLVSAVLFPLAHVGSAVPSPVPPDV
LSSILPLGLGVAGPKFFPSTFLFHLHFLFRSFDSLLLSSNGTVGASGRGLRIGV

$IP_{\beta II-C}$-12

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG**

GCCAGTCACCATGAGGCATGCGTGCGCCTGCGGCCCCAGACCTATGACCTCCAGGAGAGCAACGTGCAGC
TCAAGCTGACCATTGTGGATGCCGTGGGCTTTGGGGATCAGATCAATAAGGATGAGAGTTACAGGCCCAT
AGTTGACTACATCGATGCGCAGTTTGAAAATTATCTGCAGGAGGAGCTGAAGATCCGCCGCTCGCTCTTC
GACTACCATGACACAAGGATCCACGTTTGCCTCTACTTCATCACGCCCACAGGGCACTCCCTGAAGTCTC
TAGATCTAGTGACCATGAAGAAACTAGACAGCAAGGTGAACATTATTCCCATCATCGCCAAGGCTGACAC
CATCTCCAAGAGCGAGCTCCACAAGTTCAAGATCAAGATCATGGGCGAGTTGGTCAGCAACGGGGTCCAG
ATCTACCAGTTCCCCACGGATGATGAGGCTGTTGCAGAGATTAACGCAGTCATGAATGCACATCTGCCCT
TTGCCGTGGTGGGCAGCACCGAGGAGGTGAAGGTGGGGAACAAGCTGGTCCGAGCACGGCAGTACCCCTG
GGGAGTGGTGCAGGTGGAGAATGAGAATCACTGCGACTTCGTGAAGCTGCGGGAGATGTTGATCCGGGTG
AACATGGAAGACCTCCGCGAGCAGACCCACAGCCGGCACTACGAGCTCTACCGGCGCTGCAAGTTGGAGG
AGATGGGCTTTCACGACAGCGATGGTGACAGCCAGCCCTTCAGCCTACAAGAGACATACGAGGCCAAGAG
GAAGGAGTTCTTAAGTGAGCTGCAGAGGAAGGAGGAAGAGATGAGGCAGATGTTTGTCAACAAAGTGAAG
GAGACAGAGCTGGAGCTGAAGGAGAAGGAAAGGGAGCTCCATGAGAAGTTTGAGCACCTGAAGCGGGTCC
ACCAGGAGGAGAAGCGCAAGGTGGAGGAAAAGCGCCGGGAACTGGAGGAGGAGACCAACGCCTTCAATCG
CCGGAAGGCTGCGGTGGAGGCCCTGCAGTCGCAGGCCTTGCACGCCCCCTCGCAGCAGCCCCTGAGGAAG
GACAAGGACAAGAAGAAAGCCAGTGGCTGGTTTTCCATTTACAGTGTCACTATTCCCTGACGGAGCTGTT
ATGTGCCGCTCTAGCGAAGGCCCCAGCCGGGATGCTAGGCCTAATTGTTCAGCGTGGAGATGGCAACTCA
CGTGGTGCCCTAGGTGCAGCTGCGTGGTTTGGTATACATGCTGCAAAATTCACCCAGTTCCCCTCATTTT
AATTTTTTTAACCTACAGCTTAATTTTAATAACTTTAAAACACTTTTAAATATTTTTTTTGGCACCAGCG
TCAAGACAAATAATATCCTTTCCCATTATTTTCATAAGTAACACAGATTCCCTGATTTTTAAAAACTAAA
AATACAGCTAAACCTTTTTTATGTATAAAGTATGCCTATCATATACAGGGAGAGGGGGGTAATAAACTTC
CTGTAATGACG (1484)

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-12 amino acid sequence

ASHHEACVRLRPQTYDLQESNVQLKLTIVDAVGFGDQINKDESYRPIVDYIDAQFENYLQEELKIRRSLF
DYHDTRIHVCLYFITPTGHSLKSLDLVTMKKLDSKVNIIPIIAKADTISKSELHKFKIKIMGELVSNGVQ
IYQFPTDDEAVAEINAVMNAHLPFAVVGSTEEVKVGNKLVRARQYPWGVVQVENENHCDFVKLREMLIRV
NMEDLREQTHSRHYELYRRCKLEEMGFHDSDGDSQPFSLQETYEAKRKEFLSELQRKEEEMRQMFVNKVK
ETELELKEKERELHEKFEHLKRVHQEEKRKVEEKRRELEEETNAFNRRKAAVEALQSQALHAPSQQPLRK
DKDKKKASGWFSIYSVTIP

$IP_{\beta II-C}$-13

Reliable sequence

AAAAAAAAGAGGTTTCTTTTTAATACGACTCACTATACGCGCGCGAGCGCCGCCATGGAGTACCCATACG
ACGTACAGAACGCTCATATGGCCATGGAGGCCAGTGAATTCCACCC**AAGCAGTGGTATCAACGCAGAGTG**
GCCATTATGGCCGG

GGGGAAGTAGAGCGGAGGTGGTGGCGGCGGAGGCTTTGGCAGCTCGGGACTGAGTGCAAGAATCAGCATG
ATTCTTCAGAGGCTCTTCAGGTTCTCCTCTGTCATTCGGTCAGCCGTCTCAGTCCATTTGCGGAGGAACA
TTGGTGTTACAGCAGTGGCATTTAATAAGGAACTTGATCCTATACAGAAACTCTTTGTGGACAAGATTAG
AGAATACAAATCTAAGCGACAGACATCTGGAGGACCTGTTGATGCTAGTTCAGAGTATCAGCAAGAGCTG
GAGAGGGAGCTTTTTAAGCTCAAGCAAATGTTTGGTAATGCAGACATGAATACATTTCCCACCTTCAAAT
TTGAAGATCCCAAATTTGAAGTCATCGAAAAACCCCAGGCCTGAAGAAATAAAGTAAAATTAATCTGGTA
ATTTGTCACGGATTAGTTGTACAACTAGTTAGAAGTTTCAGAATAAACATGCATTTCATAACTGG
(487)

AAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGGGTGGGCATCGATACGGGATCCATCGAGCTCGAGCTGCCAGATG
C

$IP_{\beta II-C}$-13 amino acid sequence

GEVERRWWRRRLWQLGTECKNQHDSSEALQVLLCHSVSRLSPFAEEHWCYSSGI

$IP_{\beta II-C}$-14

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGG**

CCCCAGCCCCTGCATGGATTCCTTGTGGCTTTTCTGTCTTTTGCTAGCTTCACCAGTTTCTGTTCCTTGT
GGGATGCTGCTCTAGGGATACTCAGGGGGCTCCTGCTCTCCTTCCCCTTCCCTTCTTGCCTCACCATTCC
CCTAGGCAGGCCCTGCAGGTCCCACACTCTCCCAGGCCCTAAACTTGGGCGGCCTTGCCCTGAGAGCTGG
TCCTCCAGCGAGGCCCTGTCAGCGGTCTTAGGCTCCTGCACATGAAGGTGTGTGCCTGTGGTGTGTGGGC
TGCTCTAGGAGCAGATACAGGCTGGTATAGAGGATGCAGAAAGGTAGGGCAGTATGTTTAAGTCCAGACT
TGGCACATGGCTAGGGATACTGCTCACTAGCTGTGGAGGTCCTCAGGAGTGGAGAGAATGAGTAGGAGGG
CAGAAGCTTCCATTTTTGTCCTTCCTAAGACCCTGTTATTTGTGTTATTTCCTGCCTTTCCGAGTCCTGC
AGTGGGCTGCCCTGTACCCTGAACCTCATGAGCCTCTAAGGGAAAGGAGGAACAATTAGGACGTGGCAAT
GAGACCTGGCAGGGCAGAGTACAAGCCCAGCACCCAGTGTCCCAGCCTTACTGGGTCCTTACCCTGGGCC
AAACAGGGAGGGCTGATACCTCCTTGTTCTTCCTAGATGCCCACCTCCTACAATTTCAGCCCACAAGTCC
TTTCCACCCTAGGGGGCTTGTTGCATGGCAATAACTCATAATTTGATTTGGAGGTTTGCCCTTTACAGGG
GCAGATTTTTTGTTCAGTTCAACAATGAAATGAAGAGGAACTCCCTCTTTTTACAGCTCACTTTTATCAG
AGGCCCAGGTGCTTCAGAGCCACATTGAGTTGCTTTTTTTGGGATGAGGAAGTAGGGTTAAACTCCCCAG
TTTCCTGAGGGAGGCTCCTGACAGGTGCCCTTTGTCAGACCCTACCACAGCCTGGATAGGCAGCCACATT
GGTCCTCGCCCTTGCTCGGCACTCCGTGGTGGTCCTGCCCTTCTCCCTGCATGCCTGGGGGTTTGTTTTG
GTGTGTGAAGGTCGGTGGGTTAACTGTGTGCCTACTGAACCTGGCAAATAAACATCACCCTGCAAAGCC
(1121)

AAAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-14 amino acid sequence

PQPLHGFLVAFLSFASFTSFCSLWDAALGILRGLLLSFPFPSCLTIPLGRPCRSHTLPGPKLGRPCPESW
SSSEALSAVLGSCT

$IP_{\beta II-C}$-15

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGG**

GAAAGAGAAAAAAAAAACACTAGAATTCTTAATAGTATTGAAATAAATGTATTATATGAATATATTCAGC
ATCTCTACTGACAAAACCATTTTTAAGGACCATTGGTGGATTTTGATAGGTAAATCTTGTGCATTGCCTT
TTCTCCTCACCCATCCATCCATTCATTCACTCATTCATTTCGTATTTATTCTGTGCCAGAGACTGTGCTT
AAGGGCTAGGGATTCAGCAGTGAAAGGTGGTAAAATAGCATGTTTTCCTCAAGAAGTTAACAGTCTAGAG
AAGATGGAGCTCATAAATTCGAAAGATGGGGATGACAGGTCACATTAAAACCAGATTCAGAAGAAAAAGA
CGAAACTTGGTTTGCTTAGTACATTACTCTTTTTTGCATACATATATATAATTTGACACGCTGTTTCAAG
AAGAGATGGTACGTATCCCTTGGGTCATATCTGAGGCTGACTTGTGAGGATGTGAAGTCAGCTGATGAGC
ACATTTGGAGCCCACGCCTACTATGTGCAGATCTCTCGTCAGCGTCATTCCCAGGGCCCCAGGTGGTGTT
AAAGTCTAGGTGACTCAGACAGCTGTTCGCGTCATTCAAGCAATGAAGTCTTTTTTTTTAATTTCTTTGG
TTTAAAATTATACTCATAATTAATTGGGTTGAATTTTCCAGTGGCTTGGTTACCATAGACTTCAGTTTAT
TAGGGAACTGCTATCTGCCACTGGTTTATTATTTGCCCCAAGGTGGACTCTAAAACTTTAGGTAGGAGAC
TCTTGGTGATCAAACTGAAACTCTTGCATCTCAACCTATGAGCCGCACTTTATTGTTATTTTATTTTTTT
AGAGACAGGGTCTAGCTTTGTTGCCGAGGCTGGGGTGCAGTGGCATGATCACAGCCCACTGTAGCCTTGA
ACTCCAGGGCTCAAGTGATCCTCCCCCCTCAGCCTCCAAGTAGCTCGGACTACAGGCATGTGCCACTGCA
CCCAGCTCAAGAGTTACACTTCAAAGCACAGAATGAAAACCTATTTTTAAAGCCAACTTGATACATAGAG
TAGCTTACCAAGAATTAGTAACAACAACAAC (1083)

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-15 amino acid sequence

EREKKNTRILNSIEINVLYEYIQHLY

$IP_{\beta II-C}$-16

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCA**

CAAGCTTGGTCTAGGACTGGAATTTCAAGCATAAATGAATACTGTACAATTGTTTAATTTTAAACTATTT
TGCAGCATAGCTACCTTCAGAATTTAGTGTATCTTTTAATGTTGTATGTCTGGGATGCAAGTATTGCTAA
ATATGTTAGCCCTCCAGGTTAAAGTTGATTCAGCTTTAAGATGTTACCCTTCCAGAGGTACAGAAGAAAC
CTATTTCCAAAAAAGGTCCTTTCAGTGGTAGACTCGGGGAGAACTTGGTGGCCCCTTTGAGATGCCAGGT
TTCTTTTTTATCTAGAAATGGCTGCAAGTGGAAGCGGATAATATGTAGGCACTTTGTAAATTCATATTGA
GTAAATGAATGAAATTGTGATTTCCTGAGAATCGAACCTTGGTTCCCTAACCCTAATTGATGAGAGGCTC
GCTGCTTGATGGTGTGTACAAACTCACCTGAATGGGACTTTTTTAGACAGATTTTCATGACCTGTTCCCA
CCCCAGTTCATCATCATTTTTTTTACACCAAAAGGTTTGCAGGGTGTGGTAACTGTTTTTTTTGTGCCAT
TTTGGGGTGGAGAAGGGGGATGTGATGAAGCCAATAATTCAGGACTTATTCCTTTTTGTGTTGTGTTTTT
TTTGGCCCTTGCCCCAGAGTATGAAATAGCTTCCAGGAGCTCCAGCTATAAGCTTGGAAGTGTTTGTGTG
ATTGTAATCACATGGTGACAACACTCAGAATTTAAATTGGACTTTTGTTGTATTCTCCCCACTCAATTTG
TTTTTTAGCAGTTTAATGGGTACATTTTAGAGTCTTCCATTTTGTTGGAATTAGATCCTCCCCTTCAAAT
GCTGTAATTAACAACACTTAAAAAAACTTGAATAAAATATTGAAACCCC (889)

AAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-16 amino acid sequence

QAWSRTGISSINEYCTIV

$IP_{\beta II-C}$-17

Reliable sequence

GCGAGCGCCGCCATGGAGTACCCATACGACGTACCAGATTACGCTCATATGGCCATGGAGGCCAGTGAAT
TCCACCC**AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGGGGG**

ATTTTACCAGAGGGAGCCAGGGCTGCAGCCTCATCTGTTTGCGGATCAGAACCCGAGCTGTGCTTGTGGC
TGCGGCTGCTAACTGGCTGCGCACAGAAGCTGAGAGAAGAGGGTGGCAATAAGTACTTTTGCCTCATTCT
GAAGCCTTGGAAGGAGCTGTCACCATGCCTCACTCGTACCCAGCCCTTTCTGCTGAGCAGAAGAAGGAGT
TGTCTGACATTGCCCTGCGGATTGTAGCCCCGGGCAAAGGCATTCTGGCTGCGGATGAGTCTGTAGGCAG
CATGGCCAAGCGGCTGAGCCAAATTGGGGTGGAAAACACAGAGGAGAACCGCCGGCTGTACCGCCAGGTC
CTGTTCAGTGCTGATGACCGTGTGAAAAAGTGCATTGGAGGCGTCATTTTCTTCCATGAGACCCTCTACC
AGAAAGATGATAATGGTGTTCCCTTCGTCCGAACCATCCAGGATAAGGGCATCGTCGTGGGCATCAAGGT
TGACAAGGGTGTGGTGCCTCTAGCTGGGACTGATGGAGAAACCACCACTCAAGGGCTGGATGGGCTCTCA
GAACGCTGTGCCCAATACAAGAAGGATGGTGCTGACTTTGCCAAGTGGCGCTGTGTGCTGAAAATCAGTG
AGCGTACACCCTCTGCACTTGCCATTCTGGAGAACGCCAACGTGCTGGCCCGTTATGCCAGTATCTGCCA
GCAGAATTGGAATTGTGCCTATTGTGGAACCTGAAATATTGCCTGATGGAGACCACCACCTCCAAACGTT
GTCAGTCATGTACAGAGAGGTCTTGGCTGCTGTGTCCAGGCCCTGAGTGACCATCATGTATACCTGGAGG
GGACCTTGCTCAAGCCCAACATGGTGACCTTGGACCATGCCTGTCCCATCAAGTATTACCCCAGAGGAGA
TTGCCATGGCAACTGTCACTGCCCTGCGTCGCACTGTGCCCCCAGCTGTCCCAGGAGTGACCTTCCTGTC
TGGGGGTCAGAGCGAAGAAGAGGCATCATTCAACCTCAATGCCATCAACCGCTGCCCCCTTCCCCGACCC
TGGGCGCTTACCTTCTCCTATGGGCGTGCCCTGCAAGCCTCTGCACTCAATGCCTGGCGAGGGCAACGGG
ACAATGCTGGGGCTGCCACTGAGGAGTTCATCAAGCGGGCTGAGGTGAATGGGCTTGCAGCCCAGGGCAA
GTATGAAGGCAGTGGAGAAGATGGTGGAGCAGCAGCACAGTCACTTTACATTGCCAACCATGCCTACTGA
GTATCCACTCCATACCACAGCCCTTGGCCCAGCCATCTGCACCCACTTTTGCTTGTAGTCATGGCCAGGG
CCAAATAGCTATGCAGAGCAGAGATGCCTTCACCTGGCACCAACTTGTCTTCCTTTCTTTTTTCCCTTCC
CCTCTCTCATTGCTGCACCTGGGACCATAGGATGGGAGGATAGGGAGCCCCTCATGACTGAGGGCAGAAG
AAATTGCTAGAAGTCAGAACAGGATGGCTGGGTCTCCCCCTACCTCTTCCAGCTCCCACAATTTTCCCAT
GATGAGGTAGCTTCTCCCTGGGCTTTCCTTTTTGCCTGCCCTGTTTCCTGGGATCAGAGGGTAGTACAGA
AGCCCTGACTCATGCCTTGAGTACATACCATACAGCAAATAAATGGTAGCAAAACATTTT (1672)

AAAAAAAAAAAAAAAAAAAAAAAAAAAAA

CATGTCGGCCGCCTCGGCCTCTAGAGG

$IP_{\beta II-C}$-17 amino acid sequence

ILPEGARAAASSVCGSEPELCLWLRLLTGCAQKLREEGGNKYFCLILKPWKELSPCLTRTQPFLLSRRRS
CLTLPCGL

VITA

Akin Sevinc

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PROFESSIONAL OBJECTIVE

Poised to contribute unique blend of scientific research, and management skills to your company's value.

Results-driven research experience with a proven record in efficiency on breast cancer molecular genetics ([1] Sevinc *et al.*, 2004), molecular biology of protein-protein interactions ([2] Sevinc *et al.*, 2006; [3] Kang *et al.*, 2010; [4] Sevinc *et al.*, *accepted*), molecular and cellular biology of protein functions ([5] Sevinc *et al.*, *submitted*).

EDUCATION

Ph.D. **2004 – (ABD)**

Department of Chemistry, University of Illinois at Chicago, Chicago

M.Sc. **2001 – 2003**

Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

B.Sc. **1997 – 2001**

Department of Molecular Biology and Genetics, Middle East Technical University, Ankara, Turkey

PROFESSIONAL POSITIONS

Research Experience

Graduate Student **7/2004 – 5/2011**

Department of Chemistry, University of Illinois at Chicago, Chicago

- Seven years of scientific research experience in biochemistry, protein chemistry and molecular biology.
- Coordinate the activities of multiple researchers, organize progress across different platforms.
- Create detailed documentation of research activities, and presented studies in numerous occasions.
- Identification of protein interactions using yeast systems (two-, three-hybrid and library systems).
- Cloning, expression and purification of recombinant proteins using affinity chromatography techniques.
- Biophysical analysis of protein-protein interactions using GST-pull down and isothermal titration calorimetry.
- Handled the responsibilities of publishing the results of significant research projects.
- Goal-oriented responsibilities for developing research at expert level on several different projects;

2010, Yeast Hybrid Systems for interacting with spectrin isoforms and their impact on tetramerization.

2008, Isothermal titration calorimetry analysis for mutational effects (G46) on spectrin tetramer formation.

2005, Molecular biological and biophysical analysis of mutational effects on spectrin tetramerization site.

Research Assistant **8/2001 – 5/2004**

Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

- Three years of research experience identification of genetic determinants of cancer progression.
- Goal-oriented experience to work with skills required to analyze genetic mutations on patient cohorts.
- Worked independently and scaled up genetic analysis through to 2000 patients and 500 controls.
- Designed experiments for analyzing the impact of different genetic events on breast and prostate cancer.
- Attended 2 week course of European Genetics Foundation on Genetics Counseling.
- Worked in clinical environment in order to achieve applicability of scientific research in healthcare.
- Prepared publications of projects to present findings in scientific community.
- Goal-oriented scheduling of research activities for achievements on different projects.

2003, Analysis of the X-chromosome inactivation status and breast cancer susceptibility.

2002, RNASEL G1385A variant and breast cancer susceptibility.

2001, Chek2 1100DelC mutation and its impact on breast cancer susceptibility.

Summer Trainee

Department of Pediatrics, Ege University, Izmir, Turkey **5/1999 – 8/1999 & 5/2000 – 8/2000**

Department of Medical Biology and Genetics, Dokuz Eylul University, Izmir, Turkey **5/1998 – 8/1998**

- Participated in both clinical diagnosis and laboratory testing for prenatal diagnosis of genetic disorders.
- Presented work with two department-invited presentations to the pediatrics department.

Teaching Experience

Teaching Assistant **8/2011 – 5/2004**

Bilkent University – Department of Molecular Biology and Genetics

- Introduction to Modern Biology and General Genetics classes with laboratory sessions.

Teaching Assistant **8/2004 – 5/2011**

University of Illinois at Chicago – Department of Chemistry

- Freshman level Chemistry (I and II) courses with laboratory sessions.

PUBLICATIONS

[1] **Sevinc A.**, *et al.* Lack of association between RNASEL Arg462Gln variant and the risk of breast cancer. Anticancer Res. 24 (2004) 2547-9.

[2] **Sevinc A.**, and Fung L. W.-M. Mutational effects on brain spectrin tetramerization. FEBS Lettr. 273 (2006) 239.

[3] Kang J., Song Y., **Sevinc A.**, Fung L. W.-M. Important residue (G46) in erythroid spectrin tetramer formation. Cell. Mol. Biol. Lett. 15 (2010) 46-54.

[4] **Sevinc A.**, Witek M., and Fung L. W.-M. Yeast two-hybrid and ITC studies of alpha and beta spectrin interaction at the tetramerization site. (*accepted for publication in Cellular and Molecular Biology Letters*).

[5] **Sevinc A.**, and Fung L. W.-M. Non-erythroid beta spectrin interacting proteins and their effects on spectrin tetramerization. (*submitted for publication*).