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Research report

Mutational effects at the tetramerization site of nonerythroid alpha spectrin

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Abstract

Spectrin, a prominent cytoskeletal protein, exerts its fundamental role in cellular function by forming a sub-membrane filamentous network. An essential aspect of spectrin network formation is the tetramerization of spectrin αβ heterodimers. We used laboratory methods, the yeast two-hybrid system and random mutagenesis, to investigate, for the first time, effects of amino acid mutations on tetramerization of nonerythroid (brain) spectrin (fodrin). Based on high sequence homology with erythroid spectrin, we assume the putative tetramerization region of nonerythroid α-spectrin at the N-terminal region. We introduced mutations in the region consisting of residues 1–45 and studied mutational effects on spectrin αβ association to form tetramers. We detected single, double, and triple mutations involving 24 residues in this region. These amino acid mutations of nonerythroid α-spectrin exhibit full, partial, or no effect on the association with nonerythroid β-spectrin. Single amino acid mutations in the region of residues 1–9 (D2Y, G5V, V6D, and V8M) did not affect the association. However, seven single mutations (I15F, I15N, R18G, V22D, R25P, Y26N, and R28P) affected the αβ association. These mutations were clustered in the region predicted by sequence alignment to be crucial in nonerythroid α-spectrin for tetramerization, a region that spanned residues 12–36, corresponding to the partial domain Helix C′ (residues 21–45) in erythroid α-spectrin. In addition, two other mutations, one upstream and one downstream of this region at positions 10 (E10D) and 37 (R37P), also affected the αβ association. Our results implied nonerythroid α-spectrin partial domain helix may be longer than Helix C′ (residues 21–45 and a total of 25 residues) in erythroid α-spectrin and spanned at least residues 10–37. It is interesting to note that seven out of these nine single mutations (I15F, I15N, R18G, V22D, R25P, Y26N, R37P) were at the *a*, *d*, *e* or *g* heptad positions based on sequence alignment with erythroid α-spectrin. Four of the mutated residues (I15, R18, V22, R25) are conserved in both erythroid and nonerythroid spectrin. These positions were previously identified as hot spots in erythroid α-spectrin that lead to severe hematological symptoms. This study clearly demonstrated that single mutation in a region predicted to be critical functionally in nonerythroid α-spectrin indeed leads to functional abnormalities and may lead to neurological disorders.

Theme: Cellular and molecular biology
Topic: Gene structure and function: general

Keywords: Spectrin; Nonerythroid; Tetramerization; Yeast two-hybrid; Random mutagenesis

1. Introduction

Spectrin isoforms are filamentous, membrane-associated (cyto)skeleton proteins that are ubiquitous among vertebrate tissues and also in simple metazoans, implying that spectrin plays a fundamental role in cells [5,12,14,20,21,27]. After being first identified and purified from guinea pig erythrocytes [32], spectrin isoforms were also found in nonerythroid cells [23]. Erythroid spectrin is involved in the preservation of the unique biconcave shape of the red blood cell [1,4]. Other spectrin isoforms have been implicated in the stabilization of cell surface membranes at sites of cell–cell contacts [30], as well as in protein sorting [2] and accumulation [43], dynamics of

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nuclear architecture during mitosis, and regulation of signal transduction pathways [20].

In humans, two α-spectrin subunits (SpαI and SpαII, with "I" denoting erythroid spectrin isolated from red blood cells and "II" denoting nonerythroid spectrin), four β-spectrin subunits (SpβI, SpβII, SpβIII, and SpβIV), and a β-H spectrin have been sequenced [5]. Each α and β subunit, having molecular masses larger than 200 kDa, consists primarily of tandem homologous sequence motifs of ~106 residues that were predicted to fold into a triple α helical bundle [48]. Structural studies of these segments support the prediction. The structure of a single segment (residues 1392–1497) from *Drosophila* α-spectrin is a three-helix bundle [53]. The structure of a segment consisting two sequence motifs (residues 1771–1982) from chicken brain α-spectrin is two tandem triple helical bundles [24]. NMR solution studies of a segment (residues 1763–1872) from chicken brain α-spectrin [40] and a segment (residues 1–156) of the erythroid α-spectrin [38] also demonstrated the triple helical bundle folding of the sequence motifs.

Spα and Spβ subunits associate laterally (side-by-side) in an antiparallel fashion to form long heterodimers [49]. Two heterodimers further self-associate into tetramers $(Sp\alpha/Sp\beta)_2$ in an "end-to-end" manner. Tetramer formation involves the association of the termini of α- and β-subunits that do not participate in the dimerization process, i.e., the N-terminus of α-spectrin (αN) and the C-terminus of β-spectrin (βC). The αN/βC region is therefore referred to as the tetramerization site. One of the most fundamental functions of spectrin involves the formation of spectrin tetramers. Spectrin tetramers form a protein network (skeleton) on the inner leaflet of cell membranes. It has been suggested that the association of two partial domains (one helix from αN interacting with two helices from βC) give a complete three helical bundle [50]. Our recent NMR studies demonstrated that the N-terminal partial domain of α-spectrin consists of a single α-helix [38]. It has been implied that the C-terminal partial domain of β-spectrin consists of two helices [50].

The human erythroid spectrin $(SpαI/SpβI)_2$ is used most extensively in studying the tetramerization events. Only recently, nonerythroid spectrin began to be investigated. Studies on intact ovine brain spectrin have demonstrated that the tetramer formation of nonerythroid (brain) spectrin dimers is 15-fold higher than that of erythroid spectrin [3]. Surface plasmon resonance studies show that the binding affinity (K_d) for tetramer formation in brain spectrin segments (residues 1–145 for SpαII and 1906–2145 for SpβII) is about 5–9 nM, about 80-fold higher than that of erythroid spectrin (residues 1–154 for SpαI and 1898–2137 for SpβI with a K_d of 0.8 μM) [6]. These values are in good agreement with our isothermal titration calorimetry studies, with the K_d for SpαI 1–156 and SpβI 1898–2083 as 1.1 μM, and the K_d for SpαII 1–149 and SpβI 1898–2083 as 12 nM [34]. These data suggest that, in the process of tetramerization, the binding affinities of nonerythroid spectrin are stronger than that of erythroid spectrin.

Erythroid spectrin mutations at the tetramerization site are lead causes in a number of severe hematological diseases [11,16–18]. For example, many hereditary hemolytic anemias involve spectrin mutations that destabilize tetramer formation, resulting in abnormally high levels of dimers. Most mutations identified clinically are localized at or near the tetramerization sites, either in α- or β-spectrin. In a previous yeast two-hybrid study, a number of erythroid spectrin mutations, including several single amino acid mutations identical to clinical mutations, were identified [44]. Clinical mutations have not yet been identified at the tetramerization site for nonerythroid spectrin. However, abnormal calpain or caspase-mediated cleavage of non-erythroid brain spectrin (fodrin) has been linked to, for example, Alzheimer's disease [51], cerebral ischemia [42], and seizures [26]. Increased concentrations of IgA/IgG anti-α-fodrin in Sjogren's syndrome patients with neurological manifestations have been reported [13,33].

The yeast two-hybrid system is a transcription-based assay that relies on the principle that many proteins, including transcriptional activators, consist of multiple domains that can function independently. When individual domains are expressed separately and then brought into close proximity via non-covalent interactions, such domains can function collectively to reconstitute the activity of the intact protein. In the MatchMaker Two-Hybrid System 3 (BD Biosciences), a "bait" gene is expressed as a fusion to the GAL4 DNA-binding domain (BD), while another gene, so-called "prey", is expressed as a fusion to the GAL4 activation domain (AD) [8,15]. When "bait" and "prey" fusion proteins interact, BD and AD are brought into proximity, thus activating transcription of the reporter gene for β-galactosidase. This technology can be used to identify novel protein interactions, confirm suspected interactions, and define interacting domains, and is a well-established and well-recognized method for studying protein–protein interactions qualitatively [22]. In this study, we use this method to compare the interactions of β-spectrin with different α-spectrin mutants, generated by random mutagenesis methods. Such comparison provides, for the first time, useful information on understanding the effects of amino acid mutations on tetramerization in nonerythroid spectrin. We showed that a single amino acid mutation in SpαII may have full, partial, or no impact on the association with SpβII.

2. Materials and methods

2.1. Sequence alignment

Sequence alignment similar to that used by Mehboob et al. [34] was followed to include large regions in spectrin for alignment. Briefly, the sequence homology alignment