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Solution Structure of the RBCC/TRIM B-box1 Domain of Human MID1: B-box with a RING

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B-box domains are a defining feature of the tripartite RBCC (RING, B-box, coiled-coil) or TRIM proteins, many of which are E3 ubiquitin ligases. However, little is known about the biological function of B-boxes. In some RBCC/TRIM proteins there is only a single B-box (type 2) domain, while others have both type 1 and type 2 B-box domains in tandem adjacent to their RING domain. These two types of B-boxes share little sequence similarity, except the presence of cysteine and histidine residues: eight in most B-box1 domains and seven in B-box2 domains. We report here the high-resolution solution structure of the first B-box1 domain (from the human RBCC protein, MID1) based on 670 nuclear Overhauser effect (NOE)-derived distance restraints, 12 hydrogen bonds, and 44 dihedral angles. The domain consists of a three-turn α-helix, two short β-strands, and three β-turns, encompassing Val117 to Pro164, which binds two zinc atoms. One zinc atom is coordinated by cysteine residues 119, 122, 142, 145, while cysteine 134, 137 and histidine 150, 159 coordinate the other. This topology is markedly different from the only other B-box structure reported; that of a type 2 B-box from *Xenopus* XNF7, which binds a single zinc atom. Of note, the B-box1 structure closely resembles the folds of the RING, ZZ and U-box domains of E3 and E4 ubiquitin enzymes, raising the possibility that the B-box1 domain either has E3 activity itself or enhances the activity of RING type E3 ligases (i.e. confers E4 enzyme activity). The structure of the MID1 B-box1 also reveals two potential protein interaction surfaces. One of these is likely to provide the binding interface for Alpha 4 that is required for the localized turnover of the catalytic subunit of PP2A, the major Ser/Thr phosphatase.

Keywords: MID1; B-box; ubiquitin E3/E4 ligase; NMR; TRIM

Introduction

The tripartite RBCC (or TRIM) motif consists of an N-terminal RING domain (originally termed an "A box"), one or two "B-boxes" and a coiled-coil domain.[1] This RBCC domain arrangement is highly conserved, being found in all multi-cellular organisms. In humans, the RBCC domain characterizes a family of over 50 proteins, and although few have been characterized in detail, their importance is underscored by the fact that some are oncoproteins (e.g. PML, RFP and TIF1a) while others, when mutated (e.g. MID1), give rise to various congenital abnormalities. More recently, members of this large family have also been found to play regulatory roles in a variety of specific cellular processes, including sperm vesicle exocytosis and intracellular release of HIV.[2,3] Unlike the highly conserved arrangement of their N termini, RBCC proteins can have quite diverse C-terminal domain arrangements,[4] the most common being a single SPRY or B30.2 domain. Despite the growing importance of this RBCC family of proteins, the overall function of the highly conserved tripartite structure remains to be elucidated. Evidence from a number of studies has implicated the RING domain in ubiquitin

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Abbreviations used: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; RBCC, RING, B-box, coiled-coil; XNF7, *Xenopus* nuclear factor-7; GST, glutathione *S*-transferase; HSQC, heteronuclear single quantum coherence; TOCSY, total correlated spectroscopy; DTNB, 5,5′-dithio *bis*-2-nitrobenzoic acid; TRIM, tripartite motif.

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ligase function and the coiled-coil domain in multimerization, yet little is known about the function of B-box domains.[5]

B-boxes can have one of two different zinc-binding motifs. Type 1 B-boxes (B-box1) contain the following zinc-binding consensus sequence: C-X(2)-C-X(7-10)-C-X(2)-C-X(4-5)-C-X(2)-C(H)-X(3-6)-H-X(2-8)-H (C5(C/H)H2), while the consensus for type 2 B-boxes (B-box2) is: C-X(2)-4-C/H-X(7)-10-C-X(7)-C-X(2)-C-X(3)-6-H-X(2)-8-H (C(C/H)C3H2).[6,7] B-box1 domains are larger with their eight zinc-binding residues spaced differently than those found in B-box2 domains that have seven classical zinc-binding residues. Thus, these two domains essentially show no primary sequence homology that belies the similarity in the nomenclature. In RBCC proteins, a type 2 B-box is a defining feature. However, around one-quarter of all human RBCC proteins possess tandem B-boxes where the type 2 B-box is accompanied by an N-terminally positioned type 1 B-box (B1B2 arrangement).[4] B-boxes can also be found in isolation in some proteins and these are predominantly but not exclusively type 2 B-boxes.

To date, the only reported structure of a B-box was determined for the B-box2 domain from *Xenopus* nuclear factor-7 (XNF-7)[8] for which there is no known orthologue in humans. XNF-7 is a factor required for the establishment of dorsal–ventral patterning in this species. The XNF-7 B-box, which contains four cysteine and three histidine residues, adopts a compact structure with two β-strands positioned perpendicular to each other, a helical turn and a number of small turns. It reportedly binds one zinc atom employing the Cys1, His1, Cys4 and His2 residues.[8] The two uncoordinated cysteine residues (C2, C3) and a conserved aspartic acid (D) residue that is substituted with a cysteine in many TRIM proteins, are located ~3.5 Å from each other and oriented to bind a metal ion. The unliganded His37 (H3) is located on a loop that is close to the zinc binding cluster and which could easily be rotated to be in close proximity to the uncoordinated cysteine residues. However, as a result of this structure, B-box domains have been assumed to all exist as single zinc-binding domains.[8]

One of the better-characterized RBCC proteins is MID1, a 667 amino acid protein that contains two so-called B-boxes.[4,9] At its C-terminal end, MID1 shares a distinct domain arrangement with five other RBCC proteins; the C terminus consisting of a single fibronectin type III (FNIII) motif and both a PRY (pre-SPRY) and SPRY domain.[4] Mutations in MID1 have been found to underlie the X-linked form of Opitz G/BBB syndrome (OS), a developmental disorder characterized by numerous abnormalities including clefts of the lip and primary palate, cardiac structural defects, and genital anomalies.[10,11] Recent studies have revealed that MID1 predominantly associates with the microtubule (MT) cytoskeleton and this association persists throughout the cell cycle.[10,12,13] Work by three groups has provided evidence that MID1 functions as an E3 ubiquitin ligase. In this role, MID1 reportedly facilitates the ubiquitylation and subsequent degradation of the catalytic subunit of the most abundant Ser/Thr protein phosphatase, PP2A (PP2Ac), which regulates a vast array of intracellular processes.[9,14–16] This function of MID1 is likely mediated by the recruitment of Alpha 4, a PP2Ac-binding protein and negative regulator of PP2A activity, by the B-box1 domain and an as yet unidentified E2 ubiquitin-conjugating enzyme by the RING domain.[9,15,16] Interestingly, the activity of MID1 itself may be regulated by PP2A, since it has recently been shown to be the target of a Ser/Thr MAP kinase, presumably at the consensus recognition site (P-N-S96-P) located between the RING and B-box1 domains.[16] The direct interaction between Alpha 4 and the MID1 B-box1 domain was the first demonstration of an independent function for a B-box1 motif.[9,15]

Here we report the solution structure of the MID1 B-box1 domain, the first such B-box1 structure, and show that, unlike the reported XNF-7 B-box2, this B-box type coordinates two zinc atoms. Strikingly, we find that the type 1 B-box adopts a classical RING-like fold with conserved structural features, despite the lack of conservation of coordinating zinc residue positioning, raising the possibility of this domain possessing ubiquitin ligase activity.

Results

B-box1 expression and purification

To simplify purification, the MID1 B-box1 was expressed as an N-terminal glutathione *S*-transferase (GST)-fusion protein. While a small percentage of GST-B-box1 was found to be soluble when the bacteria were grown in Luria-Broth (LB), greater than 95% of the protein formed inclusion bodies in cultures grown in M9 minimal media, which is required to obtain ^{15}N and ^{13}C-labeled protein for NMR studies. Most attempts to increase solubility by varying growth conditions were unsuccessful. The most successful purification protocol was obtained by lysing cells in the presence of an alkyl anionic detergent, *N*-laurylsarcosine (Sarkosyl), which effectively solubilized all GST-B-box1.[17] Success of this protocol was aided by supplementing the M9 media with 440 mM sorbitol and 1.5 mM betaine. These osmolytes are hypothesized to increase the water content in the cells, minimizing aberrant folding of protein that might expose more hydrophobic surfaces and thus promote their aggregation. Despite its solubility, GST-B-box1 only bound GSH Sepharose in the presence of 4% Triton-X100 and 40 mM Chaps. While the efficiency of binding to the GSH Sepharose varied between 10–30%, we serendipitously discovered that both GST and GST-B-box1 bound benzamidine Sepharose 4FF (normally employed to bind and capture thrombin only) with ~75–90% efficiency (B.N.S. & M.A.M., unpublished results). After the resin was