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Communication

Molecules and Cells

Backbone ^{1}H, ^{15}N, and ^{13}C Resonance Assignments and Secondary Structure of the Tollip CUE Domain

Hugo F. Azurmendi[1], Sharmistha Mitra[2], Iriscilla Ayala[2], Liwu Li[3], Carla V. Finkielstein[4], and Daniel G.S. Capelluto[2,*]

The Toll-interacting protein (Tollip) is a negative regulator of the Toll-like receptor (TLR)-mediated inflammation response. Tollip is a modular protein that contains an N-terminal Tom1-binding domain (TBD), a central conserved domain 2 (C2), and a C-terminal coupling of ubiquitin to endoplasmic reticulum degradation (CUE) domain. Here, we report the sequence-specific backbone ^{1}H, ^{15}N, and ^{13}C assignments of the human Tollip CUE domain. The CUE domain was found to be a stable dimer as determined by size-exclusion chromatography and molecular cross-linking studies. Analysis of the backbone chemical shift data indicated that the CUE domain exhibits three helical elements corresponding to 52% of the protein backbone. Circular dichroism spectrum analysis confirmed the helical nature of this domain. Comparison of the location of these helical regions with those reported for yeast CUE domains suggest differences in length for all helical elements. We expect the structural analysis presented here will be the foundation for future studies on the biological significance of the Tollip CUE domain, its molecular interactions, and the mechanisms that modulate its function during the inflammatory response.

INTRODUCTION

Microbial sensing pathways are used by the vertebrate immune system to activate microbial defense mechanisms. There are two categories of immunity in vertebrates, innate and adaptive, which protect the host from infections. Whereas innate immunity detects invariant forms of microorganisms, adaptive immunity is specific and is mediated by antigen receptors found at the surface of T and B lymphocytes (Iwasaki and Medzhitov, 2010). The Toll-like receptors (TLR) are the best-studied group of innate immunity receptors that detect invading pathogens by what is referred to as pathogen-associated molecular patterns (PAMPs) (Iwasaki and Medzhitov, 2010). There are at least ten human TLR family members, each specific to a particular PAMP. These TLRs are localized in different subcellular compartments including plasma membrane, endosomes, lysosomes, and endolysosomes (Barton and Kagan, 2009). All members share the same modular architecture: *i)* an extracellular leucine-rich repeat-containing ectodomain region, which mediates contact with PAMPs, *ii)* a transmembrane region, and *iii)* a cytosolic tail that contains tandems of intracellular Toll-interleukin 1 (IL-1) receptor domains required for downstream signaling (Barton and Kagan, 2009). Although the mechanism that triggers TLR signaling activation is unknown, subsequent events result in the recruitment of one or more adaptor proteins, a process mediated by the cytosolic tail of TLRs. Downstream events promote the activation of kinases including the IL-1 receptor-associated kinases (IRAKs) 1, 2, M, and 4 that act upon their transcription factor targets to influence the expression of genes involved in the innate immune response (Gan and Li, 2006).

The Toll-interacting protein (Tollip) negatively regulates TLR signaling by interacting directly with IRAK proteins (Zhang and Ghosh, 2002). The current model proposes that, in resting cells, Tollip associates to and prevents IRAK activation by autophosphorylation. After TLR activation, IRAK autophosphorylates and subsequently phosphorylates Tollip, thus, releasing IRAK for signaling (Burns et al., 2000; Zhang and Ghosh, 2002).

Tollip is modular in nature containing an N-terminal Tom1-binding domain (TBD), a central conserved domain 2 (C2), and a C-terminal coupling of ubiquitin to endoplasmic reticulum degradation (CUE) domain (Burns et al., 2000). Tollip has been shown to selectively bind to phosphatidylinositol-3-phosphate and phosphatidylinositol-3,4,5-triphosphate (Li et al., 2004), most likely through its C2 domain. The CUE domain is a mono-ubiquitin-binding domain (Shih et al., 2003), which is found in proteins of trafficking pathways. Interestingly, the Tollip CUE domain is phosphorylated by activated IRAK proteins, an event that is believed to be necessary for IRAK's release from Tollip and to signal downstream effectors (Zhang and Ghosh, 2002). Homologous CUE domains exhibit distinct features that establish the oligomerization state of relevance for ubiquitin binding. The tertiary structure of the yeast Vps9p CUE domain reveals a dimeric helical structure enclosed around ubiquitin (Prag et al.,

[1]Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA, [2]Protein Signaling Domains Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA, [3]Laboratory of Innate Immunity and Inflammation, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA, [4]Integrated Cellular Responses Laboratory, Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
*Correspondence: capellut@vt.edu

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2003). Unlike Vps9p CUE, the CUE2-1 domain of the yeast Cue2 protein exhibits a monomeric structure and forms tight heterodimers with ubiquitin (Kang et al., 2003).

Here, we report the first structural analysis of a mammalian CUE domain, including its oligomeric state, sequence-specific backbone resonance assignments, secondary structure prediction, and homology modeling of the human Tollip CUE domain. Findings from our work will help to elucidate the regulatory role of Tollip domains in modulating intracellular signaling.

MATERIALS AND METHODS

Cloning, expression, and purification of the Tollip CUE domain

The human Tollip CUE domain cDNA (residues 219-272) was cloned into a pGEX6P1 vector (GE Healthcare) and expressed in *E. coli* (Rosetta; Stratagene). Cells were grown in Luria-Bertani media at 37°C until they reached an optical density of ~0.8. Induction of the glutathione S-transferase (GST)-Tollip CUE domain fusion protein resulted from the addition of 1 mM isopropyl thio-β-D-thiogalactopyranoside followed by 4 h incubation at 25°C. Cell pellets were suspended in cold buffer containing 50 mM Tris-HCl (pH 7.3), 500 mM NaCl, 500 mM benzamidine, 0.1 mg/ml lysozyme, 5 mM dithiothreitol (DTT), and 0.1% Triton-X-100. Suspensions were further processed by sonication, centrifuged, and the supernatants loaded onto a Glutathione Sepharose 4B (GE Healthcare) column. The GST tag was removed by incubation of the fusion protein with prescission protease (GE Healthcare; 0.04 U/μg protein) overnight at 4°C. The CUE domain was collected in a buffer containing 20 mM Tris-HCl (pH 8) and 1 M NaCl, concentrated using a 3 kDa cut-off concentrator device (Millipore) and further purified by FPLC using a size-exclusion chromatography column (Superdex 75; GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 8), 1 M NaCl, and 1 mM DTT. Protein peak fractions were pooled, exchanged in 20 mM sodium phosphate buffer (pH 7), and further concentrated for structural analysis.

Chemical cross-linking

Chemical cross-linking of the Tollip CUE domain was performed in the presence of bis(sulfosuccinimidyl) suberate (BS^3) as described (Capelluto et al., 2002). Briefly, protein (10 μM) was incubated with BS^3 (1 mM) in 100 mM HEPES (pH 7.5) for 1 h at room temperature. Reactions were stopped by the addition of Laemmli buffer. Samples were resolved by SDS-PAGE and visualized using Coomassie Blue staining.

NMR spectroscopy

NMR samples containing 1 mM of uniformly ^{15}N, ^{13}C-labeled Tollip CUE domain were prepared in 90% H_2O/10% ^2H_2O, and 20 mM sodium phosphate (pH 7). Triple-resonance experiments were acquired at 25°C using a Bruker Avance III 600 MHz spectrometer (Virginia Polytechnic Institute and State University) equipped with an inverse detected TXI probe with z-axis pulse field gradients. ^1H chemical shifts were referenced using sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) (500 μM) as an internal reference. Sequential assignments of the backbone ^1H, ^{13}C, and ^{15}N resonances were made from ^1H, ^{15}N-HSQC, CBCA(CO)NNH, HNCACB, and HNCO experiments (Grzesiek et al., 1993; Muhandiram and Kay, 1994). All spectra were processed by NMRpipe (Delaglio et al., 1995), and ^1H, ^{15}N, and ^{13}C, resonance assignments determined using the CCPNMR package (Vranken et al., 2005). The secondary structure of the protein was derived from the backbone dihedral angles ϕ and ψ predicted using the DANGLE algorithm (Cheung et al., 2010).

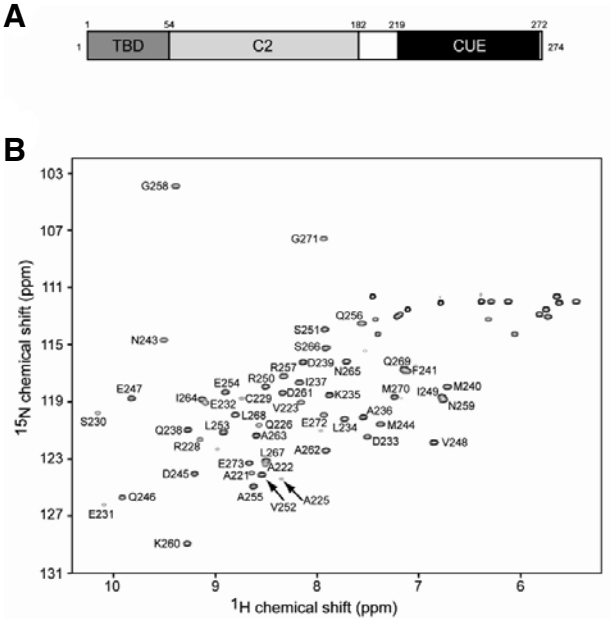

Fig. 1. (A) Schematic representation of Tollip primary structure showing the location of the TBD, C2, and CUE domains in the protein. (B) Two-dimensional ^{15}N, ^1H HSQC spectrum of the human Tollip CUE domain. Selected resonances are labeled with the corresponding residue numbers found in Tollip.

Circular dichroism spectroscopy

Far-UV CD spectra were recorded on a Jasco J-720 spectropolarimeter. Experiments were carried out in a 1 mm path-length quartz cell at 20°C. The Tollip CUE domain (5 μM) was in 20 mM sodium phosphate (pH 7). Spectra were obtained from five accumulated scans from 190 to 260 nm using a bandwidth of 1 nm and a response of 4 s at a scan speed of 20 nm/min. Spectra were deconvoluted to estimate secondary structure content with the online server DICHROWEB (Whitmore and Wallace, 2004) using the CDSSTR algorithm (Sreerama and Woody, 2004).

RESULTS AND DISCUSSION

The C-terminal human Tollip CUE domain (Fig. 1A) exhibits a well-defined resonance dispersion in the ^{15}N, ^1H heteronuclear single quantum coherence (HSQC) spectrum (Fig. 1B), which indicates that the protein is folded at the NMR scale. The number of resonances identified in the HSQC spectrum corresponds to a single conformation of the protein. Size-exclusion chromatography analysis suggests that the human Tollip CUE domain is a stable dimer with a calculated molecular mass of 15.5 kDa (Fig. 2A). We also estimated the hydrodynamic dimensions of the Tollip CUE domain as a probe for elucidating compactness of its tertiary structure (Uversky, 1993). The Stokes radius calculated for an apparent mass of 15.5 kDa is 19.6 ± 0.1 Å. This value is close to the predicted Stokes radius of a globular CUE domain (13.2 kDa), which is calculated to be 18.5 ± 0.2 Å. The dimeric state of the Tollip CUE domain was further confirmed by cross-linking the monomers of the protein with BS^3 (Fig. 2B), a chemical cross-linker that covalently interacts with primary amines that are about 11 Å apart. The stable dimeric conformation of the Tollip CUE domain is in contrast to

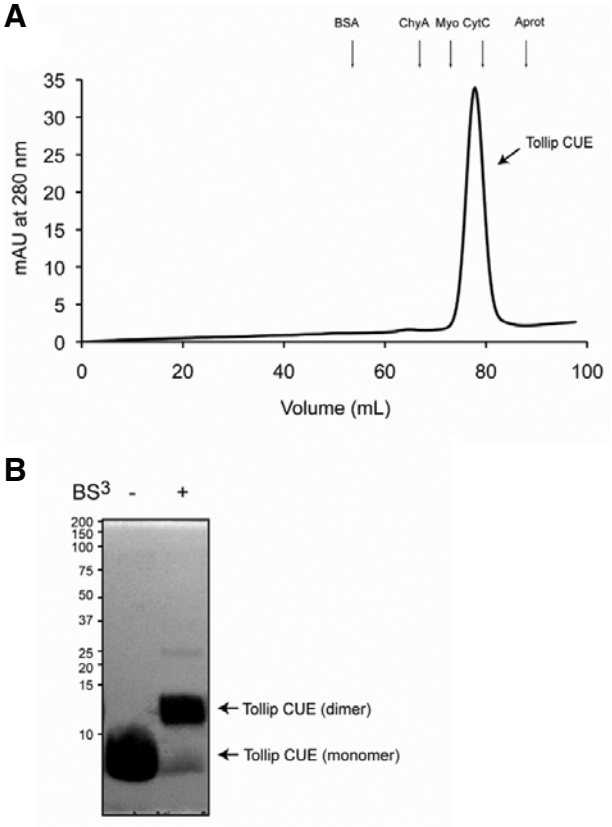

Fig. 2. Oligomeric state of the Tollip CUE domain. (A) Size-exclusion chromatography (Superdex 75) of the purified Tollip CUE domain. Protein standards were as follows: bovine serum albumin (BSA, 67 kDa), chymotrypsinogen A (ChyA, 25 kDa), myoglobin (Myo, 17 kDa), cytochrome C (CytC, 12.5 kDa), and aprotinin (Aprot, 6.5 kDa). Arrows indicate the elution volume of each molecular mass marker. (B) Molecular cross-linking with BS^3 of purified CUE domain. Samples were resolved by SDS-PAGE and visualized using Coomassie Blue staining.

the observed monomer-dimer equilibrium of the yeast Vps9p CUE domain (Prag et al., 2003). The appearance of Vps9p dimer organization, which can be applied to the human Tollip CUE domain, is suggested to be a result of domain swapping (Liu and Eisenberg, 2002) in which helix 3 of the dimeric CUE domain is exchanged between monomers (Prag et al., 2003). The relevance of Vps9p CUE's oligomeric state to its function was uncovered by sedimentation equilibrium centrifugation and site-directed mutagenesis studies (Prag et al., 2003). Findings show that the dimeric form of Vps9p CUE domain exhibits a 1,000-fold increase in binding affinity to ubiquitin when compared to the monomeric conformation. This event likely results from the direct interaction of the ligand with helix 2 of the Vpsp9 CUE domain, a structural feature that only arises as a result of dimer formation (Prag et al., 2003). Accordingly, mutations within helix 2, *i.e.*, Leu427, results in a protein that is incapable of binding to ubiquitin and, thus, establishes the relevance of the oligomeric state for Vps9p activity (Prag et al., 2003). The Vps9p/ubiquitin complex is required for efficient transport of the α-factor receptor (Ste3p) from the plasma membrane to the vacuolar compartments, where it is degraded (Davies et al., 2003). Likewise, the Tollip CUE domain is necessary for endosomal IL-1 receptor degradation (Brissoni et al., 2006), al-

Table 1. Chemical shifts of ^{1}HN, ^{15}N, ^{13}CO, ^{13}Cα, and ^{13}Cβ of the Tollip CUE domain. All chemical shifts were referenced relative to the frequency of the methyl proton resonance of DSS.

Residue	^{1}HN	^{15}N	^{13}CO	^{13}Cα	^{13}Cβ
219Pro			ND	ND	ND
220Pro			ND	63.01	31.95
221Ala	8.33	124.10	177.32	52.39	19.40
222Ala	8.26	123.37	177.65	52.21	19.42
223Val	8.08	119.13	175.79	62.55	32.71
224Asn	8.43	121.92	174.45	53.16	38.96
225Ala	8.18	124.47	177.09	52.39	19.50
226Gln	8.29	120.72	ND	53.88	29.02
227Pro			176.52	63.04	32.16
228Arg	8.58	121.69	175.86	56.15	31.43
229Cys	8.37	118.79	173.84	57.84	28.65
230Ser	9.08	119.85	175.45	57.54	64.19
231Glu	9.05	126.29	178.62	59.59	29.37
232Glu	8.55	119.15	179.05	59.68	28.34
233Asp	7.75	121.55	177.46	57.49	40.47
234Leu	7.86	120.28	178.46	58.63	41.55
235Lys	7.94	118.61	177.88	59.14	32.24
236Ala	7.77	120.17	180.91	55.02	18.28
237Ile	8.09	117.74	178.11	65.42	37.64
238Gln	8.63	121.07	178.29	59.42	28.90
239Asp	8.07	116.32	177.82	56.62	40.54
240Met	7.36	118.05	175.26	57.60	34.11
241Phe	7.57	116.90	ND	55.47	39.72
242Pro			ND	64.99	32.20
243Asn	8.75	114.74	175.11	53.15	38.65
244Met	7.69	120.64	175.13	54.99	34.10
245Asp	8.60	124.09	177.34	54.41	42.63
246Gln	8.96	125.79	177.61	59.84	28.83
247Glu	8.91	118.84	179.58	59.12	28.92
248Val	7.43	121.94	177.84	65.99	31.64
249Ile	7.40	119.90	177.01	66.08	37.52
250Arg	8.26	118.02	177.91	60.20	30.00
251Ser	7.96	114.04	177.41	61.65	62.90
252Val	8.27	124.16	177.54	66.57	31.59
253Leu	8.46	121.28	179.27	58.80	41.80
254Glu	8.45	118.39	180.66	59.66	29.46
255Ala	8.31	125.02	179.51	55.14	18.18
256Gln	7.78	113.59	175.73	53.95	26.74
257Arg	8.16	117.32	176.41	56.85	27.00
258Gly	8.69	104.00	173.24	45.75	
259Asn	7.38	118.85	173.94	53.13	38.98
260Lys	8.64	128.99	176.72	60.39	32.70
261Asp	8.17	118.44	178.55	57.89	40.35
262Ala	7.95	122.51	180.68	54.49	18.44
263Ala	8.30	121.45	178.34	55.37	18.57
264Ile	8.56	118.96	177.09	66.91	38.35
265Asn	7.86	116.27	177.78	57.02	38.41
266Ser	7.96	115.31	176.96	62.18	63.35
267Leu	8.25	123.21	179.66	57.99	41.05
268Leu	8.40	119.98	179.40	57.64	42.31
269Gln	7.57	116.78	177.17	57.24	28.77
270Met	7.62	118.75	176.74	57.45	33.25
271Gly	7.97	107.64	173.76	45.42	
272Glu	7.97	120.00	176.11	56.27	30.72
273Glu	8.33	123.36	ND	54.36	29.73
274Pro			ND	ND	ND

ND, not detected.

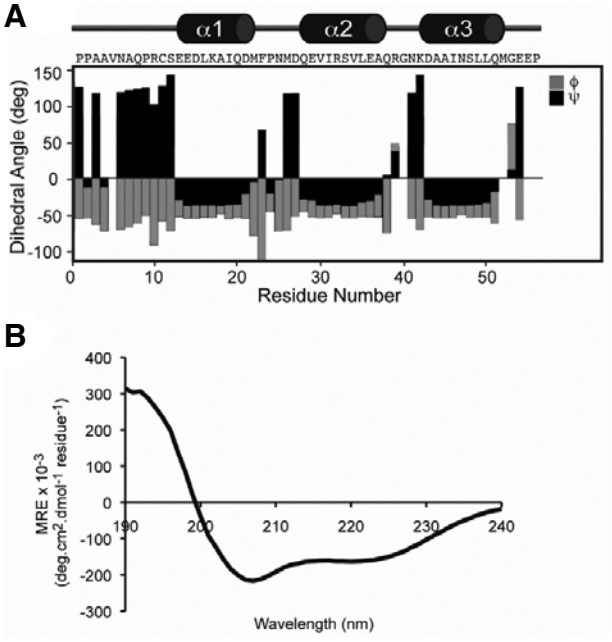

Fig. 3. Secondary structure analysis of the Tollip CUE domain. (A) Secondary structure elements of the protein based on the backbone dihedral angles as a function of the Tollip residue number. Gray bars represent predicted ϕ angles, whereas black bars represent predicted ψ angles. Predicted secondary structure elements of the Tollip CUE domain are depicted on top. (B) Far-UV circular dichroism spectrum of the Tollip CUE domain.

though the requirement of ubiquitin for this process remains to be investigated.

One hundred percent of ^{1}HN and ^{15}N of the backbone amide resonances of the protein (excluding the five Pro residues) were assigned (Fig. 1B and Table 1). In addition, 87% of carbonyls, 97% of Cα and 97% of Cβ resonances were assigned. On the basis of resonance assignments, three helical regions were defined in the Tollip CUE domain (Fig. 3A). The helices corresponded to residues Glu231-Asp239 (helix 1), Gln246-Ala255 (helix 2), and Lys260-Gln269 (helix 3). Comparison among CUE domains found in yeast Vps9p and Cue2 proteins (Kang et al., 2003; Prag et al., 2003) indicate that helix 1 is shorter in the human Tollip CUE domain. Furthermore, the Tollip CUE domain exhibits a larger helix 2 and a shorter helix 3 when specifically compared with the Vps9p CUE domain secondary structure (Figs. 3A and 4A). From our analysis, the Met-Phe-Pro and Leu-Leu hydrophobic motifs, responsible for ubiquitin binding, are expected to be located in a loop after helix 1 and at the end of helix 3, respectively (Fig. 3A). The far-UV circular dichroism spectrum of the Tollip CUE domain exhibits two minima at 208 and 222 nm and a positive signal at ~190 nm (Fig. 3B), indicative of helical structure. Prediction of the secondary structure composition shows that the Tollip CUE domain contains 59% helical structure, in close agreement with the NMR data (52%; Fig. 3A). The presence of three helix bundles in CUE domains suggest that they share structural similarities with a 40-amino acid length ubiquitin-binding ubiquitin-associated (UBA) domain (Prag et al., 2003) which also binds monoubiquitin but exhibits a greater affinity for polyubiquitins (Bertolaet et al., 2001; Wilkinson et al., 2001). Remarkably, Vps9p CUE domain also binds polyubiquitin *in vitro* (Shih et al., 2003).

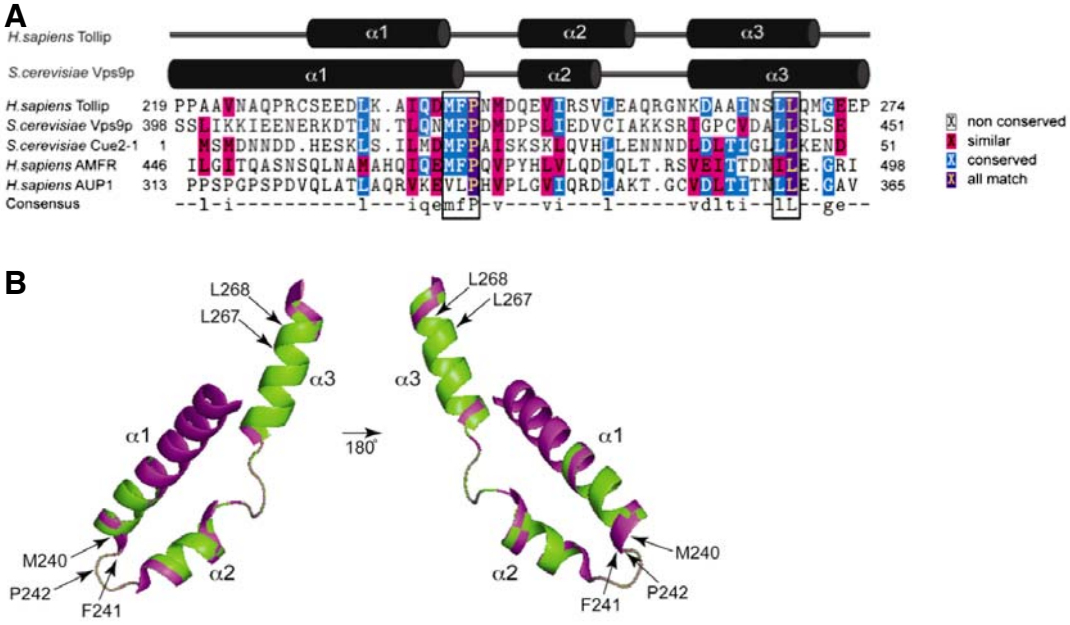

Fig. 4. Sequence conservation and structural properties of CUE domains. (A) Sequence alignment of CUE domains of human Tollip (GenBank: CAG 38508), yeast Vps9p (GenBank: AAC49314), yeast Cue2-1 (GenBank: P36075), human autocrine motility factor receptor (AMFR; GenBank: NP_001135), and human ancient ubiquitous protein 1 (AUP1; GenBank: AAH33646) reproduced from the Biology WorkBench database (http://workbench.sdsc.edu). Boxes indicate the conserved ubiquitin binding motifs found in yeast CUE domains. Secondary structures of Tollip CUE domain, determined from chemical shifts, and *Saccharomyces cere- visiae* Vps9p CUE domain are depicted above sequences. (B) Two views of the superimposed structures of Tollip CUE domain (green) and Vps9p CUE domain (magenta). Human Tollip CUE domain structure was modeled based on sequence homology to Vps9p using AL2TS (http://proteinmodel.org/AS2TS/al2ts.html) and depicted using Pymol. Ubiquitin binding residues, determined in yeast Vps9p CUE domain, are indicated on the structures.

An attempt to define the three dimensional structure of the Tollip CUE domain led to the structural determination of a domain fragment (PDB: 1WGL), albeit backbone resonance assignments of the protein were not reported. Despite the lack of information, there is a strong agreement with respect to the position of the secondary structure elements in the Tollip CUE domain and those predicted based on our NMR chemical shift data. An in depth analysis of the 1WGL tertiary structure reveals unstructured regions located at the N- and C-termini, which we believe result from the presence of seven and six additional residues translated from the vector, respectively, and that represent more than 20% of the protein domain. Instead, we have performed homology modeling of Tollip CUE using the Vps9p CUE domain as a template, a decision based on the sequence (23% identity) and functional similarities found in these two proteins. The homology modeling rendered an easy superposition of the backbones of both proteins, with helices 2 and 3 antiparallel to helix 1 (Fig. 4B). Superimposed structures aid the visualization of areas of sequence conservation, such as the Met-Phe-Pro and Leu-Leu motifs, located at the end of helix 1 and helix 3, respectively (Fig. 4B) and, thus, help to infer possible configurations of the protein-ligand complexes.

We envision using the collective data from NMR resonance assignments and homology modeling to precisely define the Tollip CUE domain molecular interactions and kinetic properties with other binding partners, *i.e.*, IRAK (Burns et al., 2000), to further understand how these associations modulate TLR downstream signaling that ultimately leads to inflammatory responses. An advantage of two-dimensional NMR chemical shift mapping over other well-characterized approaches relies in its utmost importance for the identification of yet uncharacterized ligand-binding regions while depicting molecular interactions (Pellecchia, 2005). As we show here, this technique relies on ligand binding to an ^{15}N- or ^{13}C-labeled protein, an event that leads to shifting or broadening in some cases, of specific backbone resonances of the protein. Thus, identifying the NMR chemical shift fingerprint of the Tollip CUE domain opens the possibility of mapping the binding site, and determining the stoichiometry and the kinetic and affinity values for even weak and transient ligand-binding events.

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