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Characterization of a Conserved "Threonine Clasp" in CAP-Gly Domains: Role of a Functionally Critical OH/π Interaction in Protein Recognition

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The folding and function of proteins are dependent on a multitude of intramolecular interactions of varying strengths, including salt bridges, H-bonds, and van der Waals contacts. Canonical hydrogen bonds (X−H···X, where X is an electronegative atom such as O or N) are a well-discussed and prominent contributor to protein tertiary structure and function. In addition to these classical interactions a variety of nonclassical hydrogen bonds exist in proteins involving, among others, C−H donor groups[1] or aromatic acceptor groups.[1,2] Though weaker than classical H-bonds,[3] nonclassical interactions have been predicted to make important contributions to protein structure and function. Here we provide direct evidence of a previously unreported, functionally important OH/π interaction in a family of microtubule binding protein domains.

Growth and shrinkage of microtubules (MT) are tightly controlled dynamic processes that require a complex array of regulatory proteins.[4] Plus-end tracking proteins (+TIPs) actively participate in regulating this process through a network of coordinated inter- and intramolecular associations mediated by conserved domains and motifs.[5] The CAP-Gly domain (Pfam code: 01302), found in CLIP170 and the p150^{Glued} subunit of Dynactin, forms transient interactions with EEY/F motifs located at the C-terminus of several MT-associated proteins, including α-tubulin, CLIP170, and EB1.[6]

The CAP-Gly domain adopts a 5−7 β-stranded fold (Figure 1a) with >30 examples in the Protein Data Bank (PDB). The recognition of target peptides carrying the EEY/F motif involves two highly conserved regions in CAP-Gly domains (Figure S1a): (1) the Lys and Asn residues in the GKDNG sequence that contact charged amino acids in the EEY/F motif;[6−8] and (2) a network of aromatic residues that interacts with the critical C-terminal aromatic residue of the EEY/F motif. A well-characterized example of this interaction occurs between the CAP-Gly domain of p150^{Glued} (p150CG) and the EEY/F motif of EB1 (Figure S1b).[7,9,10] A recent study of this interaction highlighted the importance of residues in the loop between β-strands 2 and 3 in p150CG.[7] Superposition of the 3D structures of 12 CAP-Gly domains deposited in the PDB (Table S1) reveals loop β2/3 has strong structural conservation (Figure S2a). In particular, the aromatic side chains of the equivalents of F52 (residue numbering consistent with 2hqh) and W57 adopt a conserved arrangement around the buried side chain of T50 (Figure 1b). Closer inspection shows the Oγ1 atom of T50 residing directly above the Phe aromatic ring. Across the 12 structures the average Oγ1/π distance is 3.7 Å and the average angle between the ring normal and a vector connecting Oγ1 to the center of the aromatic ring is 16.7° (Table S1, Figure S2b,c). Both parameters match theoretical definitions of XH/π interactions.[2] This conserved

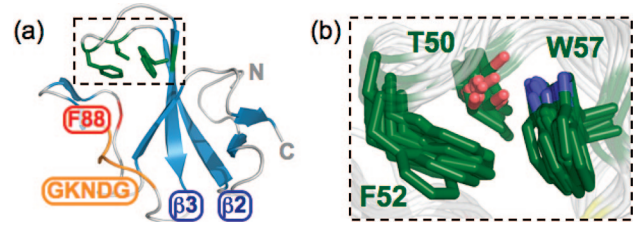

Figure 1. Structural evidence for an OH/π hydrogen bond in CAP-Gly domains. (a) The structure of the CAP-Gly domain from human p150^{Glued} (2hqh) showing functional residues. (b) Superposition of 12 CAP-Gly domain structures highlighting a Thr-Phe-Trp triad in loop β2/3.

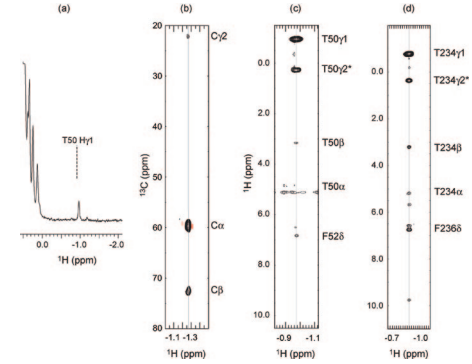

Figure 2. NMR evidence for an OH/π hydrogen bond in CAP-Gly domains. (a) 1D ^{1}H spectrum of apo-p150CG; (b) [^{1}H,^{13}C]-HMQC experiment showing intraresidue J_{HC} scalar correlations between T50-Hγ1 and side chain carbons of p150CG:EEQEEY. [^{1}H,^{1}H]-NOESYs of (c) T50-Hγ1 in apo-p150CG and (d) T234-Hγ1 in apo-ClipCG2.

architecture strongly supports the existence of an OH/π H-bond between T50 and F52 equivalents in loop β2/3 of CAP-Gly domains. Such an interaction has not been analyzed in the CAP-Gly family.

A 1D NMR spectrum of apo-p150CG shows a well-resolved upfield-shifted resonance at −0.97 ppm (Figure 2a). Selective excitation of this upfield resonance in a [^{1}H,^{13}C]-HMQC experiment yields three ^{13}C correlations that could be unambiguously assigned to the Cα, Cβ, and Cγ nuclei of T50 (Figure 2b). Strips from a [^{1}H,^{15}N]-NOESY HSQC spectrum of p150CG residues 50−53 in loop β2/3 show NOEs between backbone amide protons and T50-Hγ1 (Figure S3). A 2D [^{1}H,^{1}H] NOESY spectrum further reveals intraresidue NOEs between T50-Hγ1 and T50-Hγ2, Hβ and Hα nuclei, and an inter-residue NOE with aromatic protons (Hδ*) from F52 (Figure 2c). These NMR data allow the unambiguous assignment of the resonance at −0.97 ppm to the Hγ1 proton of T50 and confirm the close proximity of T50-Hγ1 to the aromatic ring of F52 seen in 3D structures.

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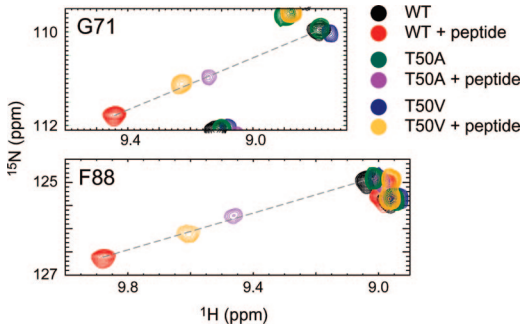

Figure 3. Mutational analysis of the functional contribution of T50 in CAP-Gly domains. [^{1}H,^{15}N]-HSQC spectra were recorded of wild-type or mutant p150CG constructs in the absence or presence of a six residue peptide derived from human EB1 (EEQEEY).

Thr Hγ1 nuclei are not commonly assigned in NMR spectra. Currently around 350 Hγ1 shifts have been deposited in the BioMagResBank (BMRB) protein chemical shift database compared to over 10800 chemical shifts for Thr Hγ2 nuclei. A chemical shift as high as -0.97 ppm has not previously been reported for a Thr Hγ1 nucleus (BMRB average: 5.14 ppm) and is indicative of a strong ring current shift. The distinctive local structure, solvent protection, and elevated shift of T50-Hγ1 observed in NMR spectra appear to result from an OH/π interaction with the aromatic ring of F52.

The side chain indole NH group of W57 also has a ^{1}H chemical shift indicative of an interaction with a delocalized electron system (8.3 ppm versus a database average of 10.1 ppm; Figure S3). Considering the structural arrangement of T50, F52, and W57 in loop β2/3 it is therefore likely that an extended H-bond network exists involving T50-Hγ1/F52-π and W57-Hϵ1/T50-Oγ1 interactions. Indeed, an NOE between W57-Hϵ1 and T50-Hγ1 is seen in a [^{1}H,^{15}N]-NOESY HSQC spectrum of p150CG (Figure S3).

The considerable sequence and structural conservation across loop β2/3 in MT-associated CAP-Gly domains (Figure S1a) suggests the Thr Hγ1/π interaction shown here in p150CG is a family wide motif. NMR spectra of the second CAP-Gly domain (ClipCG2) of CLIP170 shows a resonance at -0.80 ppm corresponding to T234-Hγ1 (the T50 homologue in ClipCG2). NOEs are detectable between this proton and F236-Hδ* (F52 homologue; Figure 2d). HSQC spectra of p150CG, ClipCG1, and CG2 show residues in loop β2/3 have near identical chemical shifts (Figure S4).

T50 in p150CG was mutated to either Val (T50V) or Ala (T50A). Comparison of [^{1}H,^{15}N]-HSQC spectra of either mutant with wild-type (wt) p150CG shows large chemical shift changes in loop β2/3 and in a structurally adjacent region (residues 88$-$91; Figure S5). Across the rest of the domain the differences are minimal. These data indicate that neither mutation disrupts the overall fold.

Both p150CG mutants retain the ability to interact with a peptide derived from the C-terminus of EB1 (sequence: EEQEEY), however with a markedly reduced affinity (Figure 3). Examination of [^{1}H,^{15}N]-HSQC spectra shows crosspeaks from the mutant proteins shift in the same direction as wt p150CG, but the magnitude of the chemical shift change at the same protein/ligand ratio is noticeably reduced. A consistent residue-by-residue trend for the magnitude of compound chemical shifts can be seen across the proteins (Figure S6). This pattern can be explained simply by considering the nature of the substitutions made at position 50. Mutation of T50 to Val replaces the γ-OH group with a methyl moiety and thereby reduces the OH/π H-bond to a lower energy CH$_3$/π interaction. Indeed, NMR spectra of T50V show an upfield shifted methyl proton resonance consistent with a CH$_3$/π interaction (data not shown). With an Ala at position 50 the pocket above the F52 aromatic ring would be unoccupied. p150CG binding affinity has a clear dependence on the side chain chemistry of residue 50. These data suggest a family wide model in which the conserved Thr in loop β2/3 in CAP-Gly domains orients the planar aromatic side chains via two H-bonds. The OH/π interaction may serve to restrict movement of the planar aromatic groups ensuring an optimal orientation to interact with EEY/F motifs.

Loop β2/3 of p150CG, and particularly A49, has been shown to interact with the coiled-coil region of EB1, which includes the C-terminal EEQEEY motif.[7] Analysis of this interaction by surface plasmon resonance reveals mutation of T50 causes a 200-fold decrease in affinity (see Supporting Information). These data suggest the H-bond network in the TxFxxxxW motif acts as a "clasp" that stabilizes the structure of loop β2/3, ensuring the optimal local conformation for interactions with EEY/F motifs and the coiled-coil region of EB1.

The TxFxxxxW motif in loop β2/3 is well, but not perfectly, conserved across the CAP-Gly family (Figure S1a). Subcategorizing the domain family based on the presence of this motif, and specifically the presence of the threonine, correlates well with reported MT-associated function. The CAP-Gly domains of the tumor suppressor CYLD do not contain a TxFxxxxW motif (Figure S1a) and have been shown to interact with proline-rich rather than EEY/F motifs.[6,11]

Several surveys of probable XH/π interactions have been reported.[1,2] The results presented here suggest that NMR spectroscopy can be a useful tool for characterizing XH/π interactions in proteins. There are several examples of upfield-shifted Thr Hγ1 resonances above 1 ppm in the BRMB. Inspection of the corresponding 3D structures reveals nearby aromatic residues that could be potential π H-bond donators. The use of tight geometric rules to identify XH/π H-bonds in 3D structures may lead to the possibility of missing genuine interactions, particularly as force fields employed in structure calculations may not favor an XH/π configuration. Upfield-shifted proton resonance frequencies for XH groups (where X = N, O, or S) could act as a useful predictor for XH/π H-bonds.

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Supporting Information Available: Material and methods, additional data and complete ref 11. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Weiss, M. S.; Brandl, M.; Suhnel, J.; Pal, D.; Hilgenfield, R. *Trends Biochem. Sci.* **2001**, *9*, 521–523.
(2) Steiner, T.; Koellner, G. *J. Mol. Biol.* **2001**, *306*, 535–537.
(3) Scheiner, S.; Kar, T.; Pattanayak, J. *J. Am. Chem. Soc.* **2002**, *124*, 13257–13264.
(4) Mitchison, T.; Kirschner, M. *Nature* **1984**, *312*, 232–234.
(5) Akhmanova, A.; Steinmetz, M. O. *Nat. Rev. Mol. Cell. Biol.* **2008**, *9*, 309–322.
(6) Weisbrich, A.; Honnappa, S.; Jaussi, R.; Okhrimenko, O.; Frey, D.; Jelesarov, I.; Akhmanova, A.; Steinmetz, M. O. *Nat. Struct. Mol. Biol.* **2007**, *14*, 959–967.
(7) Honnappa, S.; Okhrimenko, O.; Jaussi, R.; Jawhari, H.; Jelesarov, I; Winkler, F. K.; Steinmetz, M. O. *Mol. Cell* **2006**, *23*, 663–671.
(8) Hayashi, I.; Plevin, M. J.; Ikura, M. *Nat. Struct. Mol. Biol.* **2007**, *14*, 980–981.
(9) Bu, W.; Su, L. K. *J. Biol. Chem.* **2003**, *278*, 49721–49731.
(10) Hayashi, I.; Wilde, A.; Mal, T. K.; Ikura, M. *Mol. Cell* **2005**, *19*, 449–460.
(11) Saito, K; et al. *Structure* **2004**, *12*, 1719–1728.

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