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Letter to the Editor: NMR assignment of the hypothetical ENTH-VHS domain At3g16270 from *Arabidopsis thaliana*

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Biological context

Clathrin-mediated endocytosis involves several cytosolic proteins that cooperate with each other to select the content of the endocytic vesicles, and to induce the invagination, scission and release of the newly formed endocytic vesicle into the cytosol. The best-characterized components of the cytosolic endocytic machinery are the coat protein clathrin and the adaptor complex AP2. Both are structural components of the coated vesicles. In addition, several other proteins, known as 'accessory' proteins are considered to have primarily regulatory roles in the endocytosis (Wendland, 2002). ENTH (epsin N-terminal homology; Chen et al., 1998) and VHS (Vps27, Hrs and STAM; Schultz et al., 1998) domains are present in the N-terminal part of many 'accessory' proteins. Experimental evidence of their interaction with membrane phospholipids suggests for both domains a role in the first steps of the formation of the clathrin-coated vesicles.

The hypothetical ENTH-VHS domain At3g16270 from *Arabidopsis thaliana* is a 127 amino acid protein that was selected for NMR study by the RIKEN Structural Genomics/Proteomics Initiative (RSGI) (Yokoyama et al., 2000). The presence of an ENTH or a VHS domain in this hypothetical protein is predicted by distant amino acid sequence similarity.

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Methods and experiments

The Arabidopsis ENTH/VHS domain was produced as a 173 amino acid recombinant protein with an N-terminal HAT affinity tag and a TEV protease cleavage site. The ^{13}C- and ^{15}N-labeled protein was produced by the *E. coli* cell-free synthesis system (Kigawa et al., 1999). The protein was first adsorbed on a TALON Superflow affinity column using 20 mM Tris-HCl (pH 8.0) containing 1 M NaCl, and eluted with 20 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 500 mM imidazole. The HAT-tag was then removed by incubation with TEV protease with an overnight dialysis at 4 °C against 20 mM Tris-HCl (pH 8.0) containing 1 M NaCl. To remove the HAT-tag and the TEV protease from the reaction mixture, the solution was added to a TALON Superflow affinity column. The flowthrough fraction was collected and loaded onto a HiTrap SP cation exchange column equilibrated with 5 mM Tris-HCl (pH 7.0). Finally, the purified protein was eluted with a gradient of 5 mM Tris-HCl (pH 7.0) to 20 mM Tris-HCl (pH 7.0) containing 1 M NaCl.

The protein sample used for the NMR measurements comprises 140 amino acid residues including the non-native terminal sequences that were added in the cloning process. All NMR experiments were recorded with one sample of approximately 1.20 mM, uniformly ^{13}C and ^{15}N labeled protein in 20 mM Tris buffer at pH 7.5, containing 100 mM NaCl, 1 mM dith-

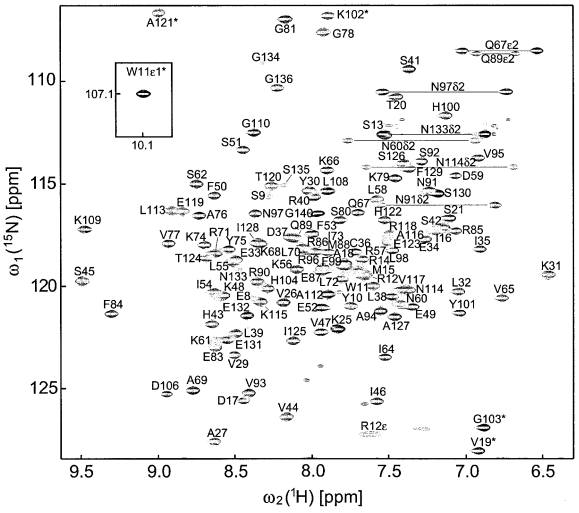

Figure 1. [^{1}H,^{15}N]-HSQC spectrum of the ENTH-VHS domain At3g16270 from *Arabidopsis thaliana* recorded at 600 MHz ^{1}H resonance frequency and 25 °C using 1.20 mM uniformly ^{13}C and ^{15}N labeled protein, 20 mM Tris buffer, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 0.02% NaN_3, 10% D_2O (v/v). Residues marked with * are folded in the ^{15}N dimension.

iothreitol, 0.02% NaN_3 and 10% D_2O (v/v). All NMR measurements were performed at 25 °C on Bruker DRX 600 and AV 800 spectrometers (for the NOESY experiments). ^{1}H, ^{15}N and ^{13}C chemical shifts were referenced relative to the frequency of the ^{2}H lock resonance of water.

Backbone and side-chain assignments were obtained by using a combination of standard triple resonance experiments (Cavanagh, 1996). 2D [^{1}H,^{15}N]-HSQC (Figure 1) and 3D HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCACB and CBCA(CO)NH spectra were used for the ^{1}H, ^{15}N and ^{13}C assignment of the protein backbone. Side-chain ^{1}H and ^{13}C assignments of the non-aromatic side-chain CH_n moieties, including all prolines, were obtained using 2D [^{1}H,^{13}C]-HSQC, 3D HBHA(CO)NH, 3D H(CCCO)NH, 3D (H)CC(CO)NH, 3D HCCH-COSY, 3D HCCH-TOCSY and 3D (H)CCH-TOCSY spectra. Assignments were checked for consistency with 3D ^{15}N-edited [^{1}H,^{1}H]-NOESY and ^{13}C-edited [^{1}H,^{1}H]-NOESY spectra recorded with 80 ms mixing time. The ^{1}H and ^{13}C spin systems of the aromatic rings of Phe, Trp and Tyr were identified using a 3D HCCH-COSY experiment. NOEs between aromatic protons and the βCH_2 group observed in the 3D ^{13}C-resolved [^{1}H,^{1}H]-NOESY spectrum were used to establish the sequence-specific resonance assignments of aromatic side-chains. The 2D and 3D spectra were processed using NMRPipe (Delaglio et al., 1995) and visualized and analyzed with the NMRView software (Johnson and Blevins, 1994).

Extent of assignments and data deposition

For the native protein of residues 8 to 134, 97% of the backbone H^N, N, H^α, C^α and C' resonances and 97% of the ^{1}H and ^{13}C shifts of the side-chain CH_n moieties were unambiguously identified and assigned. Side-chain ^{1}H and ^{15}N assignments were obtained for the indole group of W11 and all Asn and Gln side-chains. Most chemical shifts of the residues 1–7 and 137–140 in the presumably unstructured, non-native N- and C-terminal sequences and of the residues D22 and E23 could not be assigned, as well as the backbone amide groups of residues D24, S82 and D111. Among the remaining side-chain CH_n moieties of the native protein, only H^δ of K25, H^ζ/C^ζ of F50 and F84, H^γ/C^γ, H^δ/C^δ of R57 and $H^{\varepsilon 1}/C^{\varepsilon 1}$ of H100, H104 and H122 remain unassigned. The ^{1}H, ^{13}C and ^{15}N chemical shifts have been deposited in the BioMagResBank database (http://www.bmrb.wisc.edu) under BMRB accession number 5928.

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