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ARTICLE

^{1}H, ^{13}C, and ^{15}N chemical shift assignments for the Eps15-EH2-stonin 2 complex

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Abstract EH domains are protein–protein interaction domains that function in vesicular trafficking and endocytosis. Here, we report the NMR spectral assignments of the high-affinity complex between the second EH domain of Eps15 and a stonin 2 peptide—providing the basis for the characterization of a two-site binding mode.

Keywords EH domains · Eps15 · Stonin 2 · Protein–protein interactions · Endocytosis

Biological context

Eps15 has been implicated in clathrin-mediated endocytosis as an assembly factor for the endocytic protein machinery (Salcini et al. 1999). Its N-terminus comprises three Eps15 homology (EH) domains, protein interaction modules that are exclusively found in proteins involved in vesicular trafficking.

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EH domains have been shown to bind to peptide motifs containing the residues asparagine-proline-phenylalanine (NPF motifs) (Santolini et al. 1999). All interactions reported so far are of very low affinity with dissociation constants in the micro- to millimolar range and involve a single NPF motif that binds to one EH domain.

Stonin 2 is a neuronal adaptor protein that mediates the internalization of synaptic vesicles (Diril et al. 2006). Within a natively unstructured region, stonin 2 contains two NPF motifs that interact with the EH domains of Eps15. Closer inspection of the Eps15-stonin 2 interaction revealed that the interaction is of extremely high affinity with a dissociation constant in the nanomolar range (Rumpf et al. 2008). It is mediated by the second EH domain of Eps15 that binds to both of the stonin 2 NPF motifs. To characterize this novel binding mode of EH domains and elucidate how specificity is mediated, we assigned the chemical shifts of the binding partners and solved the solution structure of the Eps15-EH2-stonin 2 complex.

Methods and experiments

The second EH domain of human Eps15, amino acids 121–215 (EH2), and a human stonin 2 fragment (amino acids 301–340) were amplified and cloned into a pGEX6P1 vector (GE Healthcare) via *Bam*HI and *Xho*I restriction sites.

The proteins were expressed in the *Escherichia coli* BL21 (DE3) strain grown in LB medium. For isotope labeling M9 minimal medium was used supplemented with $^{15}NH_4Cl$ (Spectra, Stable Isotopes, Columbia, USA) or $^{15}NH_4Cl$ and 2 g/l ^{13}C-glucose (Cambridge Isotope Laboratories, Andover, USA).

Cell pellets were suspended in lysis buffer (50 mM Tris/HCl pH 7.5, 300 mM NaCl, 4 mM DTT, 4 mM Benzamidine, 1–2 mM EDTA, Protease Inhibitor cocktail (Roche)) and disrupted in a microfluidizer (Microfluidics, Newton, Massachusetts, USA). Cleared lysates were applied to glutathione Sepharose 4B (GE Healthcare) and washed with a high salt buffer. The glutathione S-transferase (GST) tag was cleaved off by incubation with PreScission protease (GE Healthcare). Proteins were further purified via gel filtration on a Superdex 75 column (GE Healthcare) and concentrated in 10 mM Tris/HCl pH 7.0, 100 mM NaCl, 2 mM DTT (and 2 mM $CaCl_2$ for the EH domain) to 20–40 mg/ml. Spectra of differentially labeled complex in 10 mM perdeuterated Tris/HCl pH 7.0 (CDN Isotopes, Pointe-Claire, Canada), 100 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT, 0.02% NaN_3 were acquired on Bruker DRX500, DRX600, AV800 and AV900 spectrometers at 295 K. All spectrometers were equipped with cryogenic triple-resonance probes. Spectra were processed with NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson and Blevins 1994). Backbone chemical shifts were assigned from HNCA, HNCACB and CBCA(CO)NH experiments at 500 MHz ^{1}H frequency. Side chain assignments were obtained from H(CCO)NH–TOCSY, (H)C(CCO)NH, H(C)CH–TOCSY, and ^{1}H–^{15}N–TOCSY (Sattler et al. 1999). Aromatic side chains were obtained from a 2D ^{1}H,^{1}H NOESY and (Hβ)Cβ(CγCδ)Hδ and (Hβ)Cβ(CγCδCε)Hε (Yamazaki et al. 1993). Proton chemical shifts were referenced with respect to residual solvent signal (4.803 ppm at 295 K), or calculated using frequency ratios of $^{15}N/^{1}H = 0.1011329118$ and $^{13}C/^{1}H = 0.251449530$ (Wishart et al. 1995). Quadrature detection in the indirect dimension of the multi-dimensional experiments was achieved by the echo/antiecho detection scheme for ^{15}N, and by the States-TPPI method for ^{1}H and ^{13}C.

Extent of assignments and data deposition

Near complete assignments could be obtained for the Eps15-stonin 2 complex. Residues at the end of the second helix of the EH domain as well as the linker to the third helix Val154-Lys159 exhibited significant line-broadening and hence several resonances could not be assigned: Leu155(CD1), Leu156(N), Asn157(HN, N, ND2, HD21, HD22). The assignments for residues within this region were mostly inferred from ^{15}N-edited and ^{13}C-edited NOESY spectra. About 97.7% of all resonances excluding residues originating from the expression vector could be assigned and backbone assignment is 99% complete. Figure 1a and b summarize the backbone assignments for the EH domain and the stonin 2 ligand, respectively. Secondary chemical shifts $\Delta\delta$(Cα-Cβ) are given in Fig. 2 a, b. In agreement with

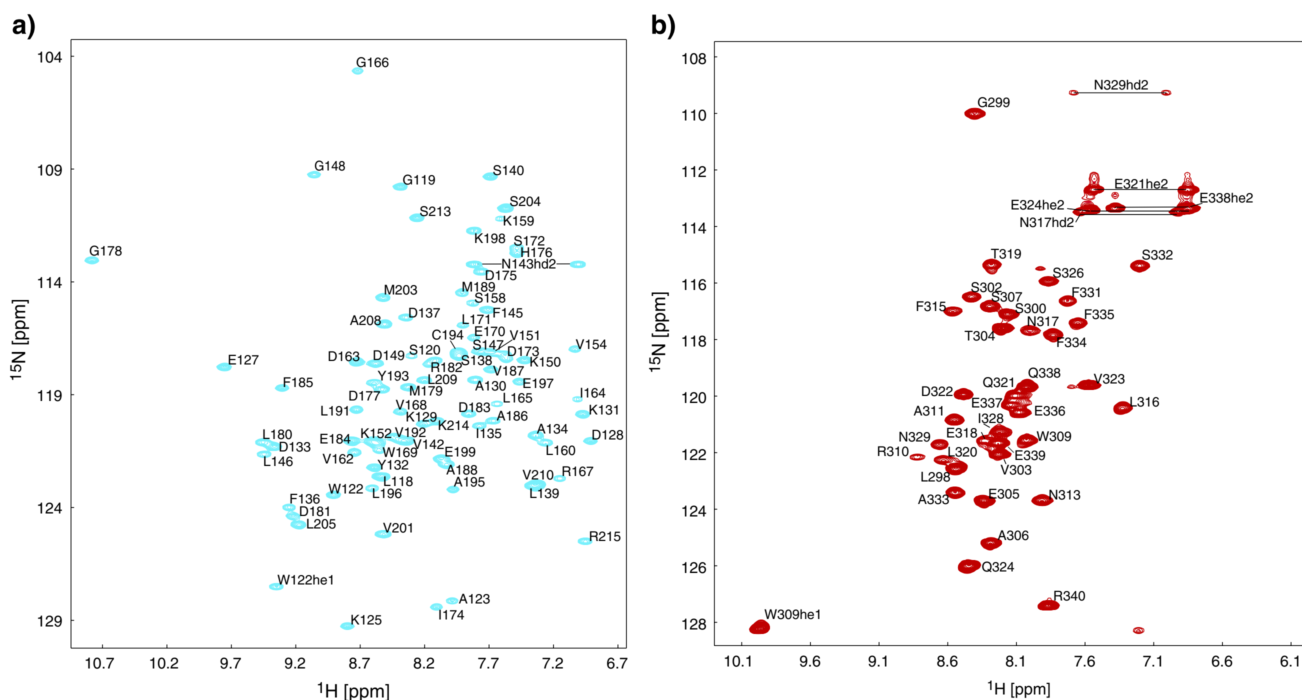

Fig. 1 (**a**) ^{1}H, ^{15}N-HSQC of the second EH domain of Eps15 in complex with unlabeled stonin 2 peptide. The spectrum was recorded on a DRX500 spectrometer at 22°C. Backbone resonance assignments are indicated in a one-letter amino acid code. (**b**) ^{1}H, ^{15}N-HSQC of stonin 2 in complex with unlabeled EH domain

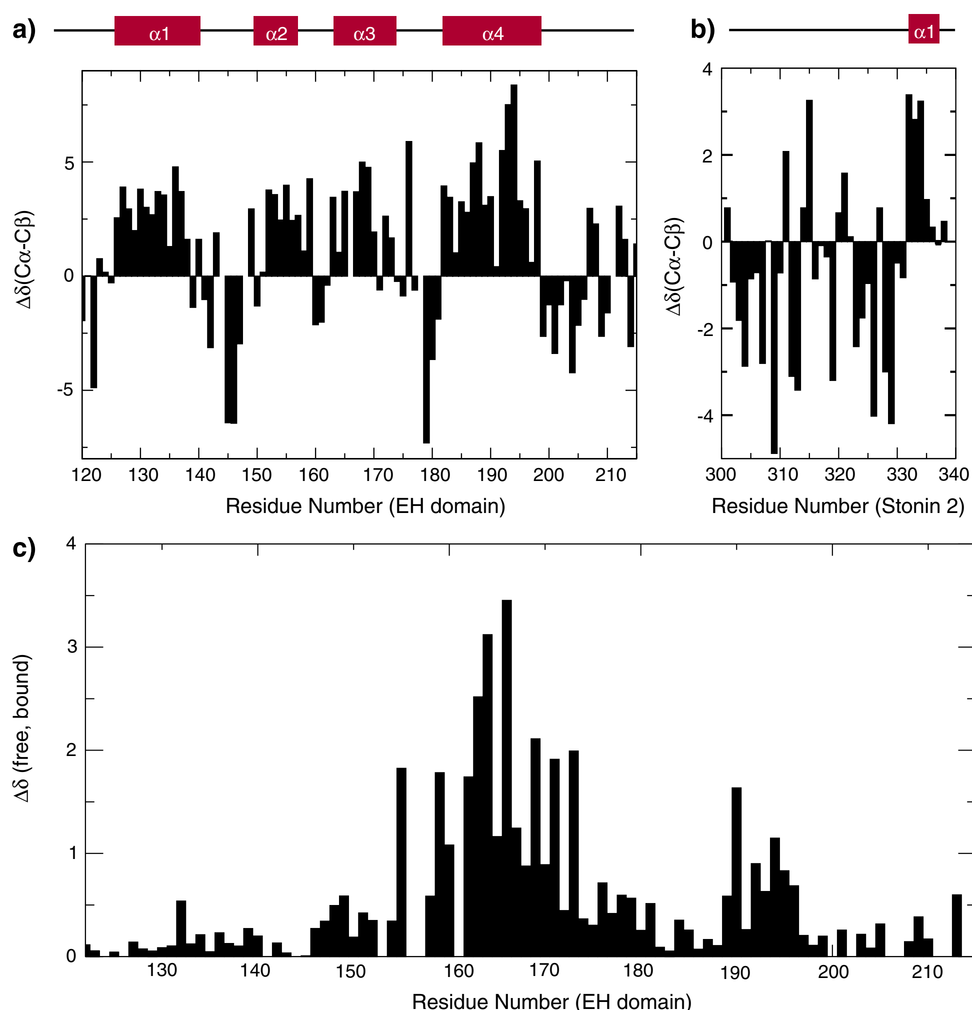

Fig. 2 (**a**) Secondary chemical shifts $\Delta\delta(C\alpha{-}C\beta)$ and secondary structure of the Eps15 EH2 domain in complex with stonin 2. (**b**) Secondary chemical shifts $\Delta\delta(C\alpha{-}C\beta)$ of the stonin 2 peptide bound to Eps15 EH2. (**c**) Differences in HN, N chemical shifts of the Eps15 EH2 domain upon binding of stonin 2. Within the sequence two regions undergo major chemical shift changes. The first region (residues 154–173) corresponds to the conserved binding pocket whereas the second region (residues 188–196) corresponds to an additional binding site. The weighted chemical shift differences are shown as calculated according to $\Delta\delta = [(4 \times \delta_{HN})^2 + \delta_N^2]1/2$. The chemical shifts of the free EH domain rely on the assignment by de Beer et al. (1998, BMRB #4184)

the structure of the EH domain, four helical regions are predicted from secondary chemical shift analysis. The stonin 2 peptide bound to the EH domain shows a small helical region at its C-terminus, otherwise secondary chemical shifts are indicative of an extended conformation.

Chemical shift differences for the free and stonin 2-bound EH domain are shown in Fig. 2c. Two regions within the sequence exhibit extensive chemical shift differences. The first region, comprising residues 154–173, can mainly be attributed to the binding site that has been described by de Beer et al. (2000). Chemical shift differences of residues 188–197 can be attributed to an additional binding site.

It is this combination of two binding sites that accounts for the exceptional affinity observed for the Eps15-stonin 2 interaction.

The assignment data of the Eps15-stonin 2 complex have been deposited at the BMRB (http://www.bmrb.wisc.edu) and can be accessed under the accession number 15554.

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