Cloning, Expression and Purification of constructs

EH domains of human Eps15, amino acids 1-120 (EH1), 121-215 (EH2), 217-313 (EH3), 1-313 (EH1-3) were amplified and cloned into a pGEX6P1 vector (GE Healthcare). Truncated versions of human stonin2 were amplified (amino acids 204-340, 301-340, 308-340, 312-332) and cloned into pGEX6P1 or pGEX4T1 vectors. 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).

All Eps15 and stonin2 constructs were purified with Glutathione Sepharose 4B (GE Healthcare) under standard conditions. The Glutathione S-transferase (GST) tag was cleaved off by incubation with PreScission protease (GE Healthcare) or Thrombin (Serva). Proteins were further purified via gel filtration on a Superdex 75 column (GE Healthcare) and concentrated in 10 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM DTT (and 2 mM $CaCl_2$ for EH domains) to 20-40 mg/ml. A construct of rat epsin1 (amino acids 496-575), comprising all three NPF motifs was cloned into a petDuet1 vector (Novagen) and purified according to (Kalthoff, 2003) with the modification that a microfluidizer was used to disrupt the cells. A construct of epsin1 comprising 2 NPF motifs (residues 491-526), was cloned into a pGEX6P1 vector. Purification of this construct was performed as described above for stonin2.

All substitutions (single or multiple amino acid changes) were made using the site directed mutagenesis kit from Stratagene according to the supplier's protocol.

Immunoprecipitation experiments

12 h after transient transfection of HEK293 FlipIn cells (inducibly expressing HA-stonin2 WT) with Flag-tagged Eps15 construct, stonin2 expression was induced by addition of 1 g/ml doxycycline. In the case of HA-stonin2 δNPF (a stonin2 mutant where the two NPF motifs have been deleted), stonin2 and Eps15 were transiently expressed in HEK293cells. 36 hours post-transfection cells were lysed in 20 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, 0.3 % mammalian protease inhibitor cocktail (SIGMA) for 10 min on ice. 2 mg (2 μg/μl) cleared cell extract was incubated with protein A/G Sepharose (Santa Cruz) coupled to polyclonal anti-stonin2 antibody or pre-immune serum as control for 4 h at 4 °C on a rotator. For immuno-precipitations from rat brain extract, brain tissue was homogenized in 320 mM sucrose, 4 mM HEPES, 1 mM PMSF, 0.3 % mammalian protease inhibitor cocktail. After 10 min centrifugation at 1000 g the supernatant was adjusted to the lysis conditions mentioned above. Following a 10 min incubation at 4 °C the supernatant was cleared by centrifugation for 15 min at 43500 g and 15 min at 265000 g. 5 mg rat brain extract (4 μg/μl) were incubated with Protein A/G sepharose coupled to polyclonal anti-Eps15 antibody (gift from P. DiFiore) or rabbit IgGs (Biomol) as control for 4 h at 4 °C on a rotator. Beads were washed extensively and proteins were eluted with sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting against stonin2 (1:500 rb anti-stonin2, Haucke-lab), Eps15 (1:100, ms anti-Eps15, BD Biosciences) or Flag (1:500, rb anti-Flag, Sigma), epsin (1:20, ms anti-Epsin, Santa Cruz) and Clathrin Heavy Chain (1:500, ms anti-CHC, TD1 clone).

Membrane recruitment assay

NIE-115 cells were co-transfected with lumenally Flag-tagged Synaptotagmin1, HA-tagged stonin2 WT or stonin2 NAV mutant and GFP-EH1-3. 24 hours post-transfection cells were fixed and surface synaptotagmin1 was labeled under non-permeabilizing conditions by anti-Flag antibodies, followed by an incubation with Alexa-647 coupled goat-anti-mouse antibodies. After permeabilization with 0.3% Triton X-100 stonin2 was labeled by anti-stonin2 antibodies which were detected by Alexa-594 coupled goat-anti-rabbit antibodies.

NMR spectroscopy

For NMR measurements the second EH domain of Eps15 and a stonin2 fragment comprising amino acids 301-340 were used. All complex spectra were recorded on differentially labeled samples, that consisted of a mixture of 1:1.1 of labeled:unlabeled protein. Data were acquired at 295 K on Bruker DRX500, DRX600, AV800 and AV900 spectrometers equipped with cryogenic triple-resonance probes. The sample buffer contained 10 mM perdeuterated Tris/HCl pH 7.0 (CDN Isotopes, Pointe-Claire, Canada), 100 mM NaCl, 2 mM $CaCl_2$, 1 mM DTT and 0.02 % NaN_3.

Spectra were processed with NMRPipe (Delaglio *et al*, 1995) and analyzed using NMRView (Johnson and Blevins, 1994). The ^{1}H, ^{13}C and ^{15}N chemical shifts were assigned by standard methods (Sattler *et al*, 1999). Distance restraints were derived from ^{15}N- or ^{13}C-resolved three-dimensional and ^{1}H homonuclear two-dimensional NOESY. Intermolecular NOEs were identified from ^{12}C, ^{14}N-filtered, ^{13}C-resolved, three-dimensional NOESY experiments (Sattler *et al*, 1999). HN-N residual dipolar couplings

(RDC) of the EH domain were measured using a spin-state-selective ^{1}H, ^{15}N correlation experiment (Cordier *et al*, 1999) in polyethylene glycol based liquid crystalline medium (Ruckert and Otting, 2000) as well as in filamentous pf1 phage medium (Profos, Regensburg, Germany). Restraints for the backbone angles ϕ and ψ were derived from TALOS (Cornilescu *et al*, 1999).

Restraints for $\chi 1$ angles of aromatic residues of the stonin2 peptide were obtained from three-bond $^{3}J\ NC_{\gamma}$ and 3 $JC'C_{\gamma}$ couplings (Hu *et al*, 1997). Slowly exchanging amide protons for the EH domain were identified from ^{1}H, ^{15}N correlation experiments after re-dissolving lyophilized protein in D_2O.

For NMR titration, increasing amounts of Epsin peptide comprising amino acids 491-526 were added to 0.05 mM ^{15}N-labeled EH2 domain up to a six-fold excess, and chemical shifts were monitored in two-dimensional ^{1}H, ^{15}N HSQC experiments.

^{15}N relaxation (T1, T2 and heteronuclear-^{15}N NOE) was measured on ^{15}N-labeled samples at 295 K and 500 MHz ^{1}H Larmor frequency, as described (Farrow *et al*, 1994). Correlation times at 295 K for EH2 free and in complex to stonin2 were determined to be 6.4 and 8.8 ns respectively. All mutants of the EH domain were found to be folded as judged by 1-dimensional ^{1}H NMR spectra.

References

Cordier F, Dingley AJ, and Grzesiek S (1999) A doublet-separated sensitivity-enhanced HSQC for the determination of scalar and dipolar one-bond J-couplings. *J Biomol NMR,* **13,** 175-180.

Cornilescu G, Delaglio F, and Bax A (1999) Protein backbone angle restraints from searching a database for chemical shift and sequence homology. *J Biomol NMR,* **13,** 289-302.

Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, and Bax A (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR,* **6,** 277-293.

Farrow NA, Muhandiram R, Singer AU, Pascal SM, Kay CM, Gish G, Shoelson SE, Pawson T, Formankay JD, and Kay LE (1994) Backbone Dynamics of A Free and A Phosphopeptide-Complexed Src Homology-2 Domain Studied by N-15 Nmr Relaxation. *Biochemistry,* **33,** 5984-6003.

Hu JS, Grzesiek S, and Bax A (1997) Two-dimensional NMR methods for determining (chi 1) angles of aromatic residues in proteins from three-bond J(C'C gamma) and J(NC gamma) couplings. *Journal of the American Chemical Society,* **119,** 1803-1804.

Johnson BA and Blevins RA (1994) Nmr View - A Computer-Program for the Visualization and Analysis of Nmr Data. *Journal of Biomolecular Nmr,* **4,** 603-614.

Ruckert M and Otting G (2000) Alignment of biological macromolecules in novel nonionic liquid crystalline media for NMR experiments. *Journal of the American Chemical Society,* **122,** 7793-7797.

Sattler M, Schleucher J, and Griesinger C (1999) Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. *Progress in NMR Spectrosc,* **34,** 93-158.