Dimeric Endophilin A2 Stimulates Assembly and GTPase Activity of Dynamin 2

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Supporting Material

METHODS

Generation of endophilin constructs. cDNA of mouse endophilin A1(gi:31560792) and endophilin A2 (gi:17390906) were generated by polymerase chain reaction (PCR) from a mouse brain cDNA library (donated by Dr. E. Ross, UT Southwestern Medical Center) using primers containing restriction sites BamHI at N terminus (5') and HindIII and KpnI (C terminus, 3') for endophilin A1 and endophilin A2, respectively. The products were subcloned into a pQE-80-L vector (containing 6 His) using cloning sites BamHI and HindIII for endophilin A1 and Sph I and Sal I for endophilin A2, sequenced, and used for expression in *E. coli*. The pQE-BAR domain construct, which contains amino acid residues 1-268 of endophilin A2, was obtained by digestion of full length pQE-endophilin 2 with SmaI and self-ligation. Endophilin A1 in pCMVmycEGFP was subcloned into a pQE-80-L vector using cloning sites SphI (N terminus, 5') and SalI (C terminus, 3'). The EGFP-endophilin A2 construct was obtained by subcloning full length endophilin A2 from the pQE vector into pEGFP-C1 vector by PCR using primers containing restriction sites of Bgl II (N-terminal, 5') and of EcoR I (c-terminal, 3'). The EGFP-BAR domain construct was generated by deletion of the fragment between two Sma I sites from full length EGFP-endophilin 2 and self-ligation. cDNAs of EGFP, EGFP-endophilin A2, and EGFP-BAR domain were used as templates to make an A206K point mutation in EGFP in these three constructs. This mutation reduces the slight propensity of EGFP to dimerize (1). These mutants were produced using the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer's protocol.

Purification of recombinant endophilins. His_6-endophilin A1, His_6-myc-EGFP endophilin A1, and His_6-endophilin A2 were expressed in *E. coli*. Bacterial cells were resuspended in lysis buffer containing 20 mM HEPES pH 8.0, 0.1 M NaCl, 1 mM β-mercaptoethanol, a protease inhibitor cocktail (0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml each of N α-p-tosyl-L-lysine chloromethyl ester, N α-p-tosyl-L-arginine methyl ester, N α-p-tosyl-L-lysine chloromethyl ketone, leupeptin, and pepstatin A), and lysozyme (0.05 mg/ml). The cell suspension was sonicated and centrifuged at 100,000 × g for 45 min at 4°C. The supernatants were mixed with Ni^{2+}- NTA resin for 1 hour at 4°C. The resin was washed with lysis buffer supplemented with 30 mM imidazole pH 8.0 and 0.3 M NaCl, and His_6-endophilin was eluted with lysis buffer supplemented with 150 mM imidazole pH 8.0. The purified proteins were dialyzed against 20 mM HEPES pH 7.5, 0.1 M NaCl, 0.5 mM DTT, and 0.2 mM PMSF. Aliquots of the protein were frozen in liquid N_2.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were carried out in a Beckman XL-I analytical ultracentrifuge using absorption optics at 280 nm. Samples were centrifuged at 12,000 and 19,000 rpm at 4°C in an An60Ti rotor using double sector or 6-channel centerpieces with optical pathlength of 1.2 cm. Data were collected by 0.001 cm stepwise scanning, with each data set representing the average of 5 scans. Baselines were obtained from 42,000 rpm overspeed runs. The calculated partial specific volume of endophilin A2 is 0.726 cm^3g^{-1} at 4°C and its calculated molecular mass is 42,916 Da. The solvent density was 1.004 g/ml at 4°C. The experiments were performed in buffer containing 20 mM HEPES pH 7.0, 10 mM EDTA, 100 mM NaCl, 1 mM DTT, and 0.2 mM PMSF. Data were fit using Beckman Optima XL-A/XL-I software, employing "ideall" models for single-species fits and "assoc4" models to obtain monomer/n-mer equilibrium constants for up to four interacting species.

SUPPORTING MATERIAL FIGURES

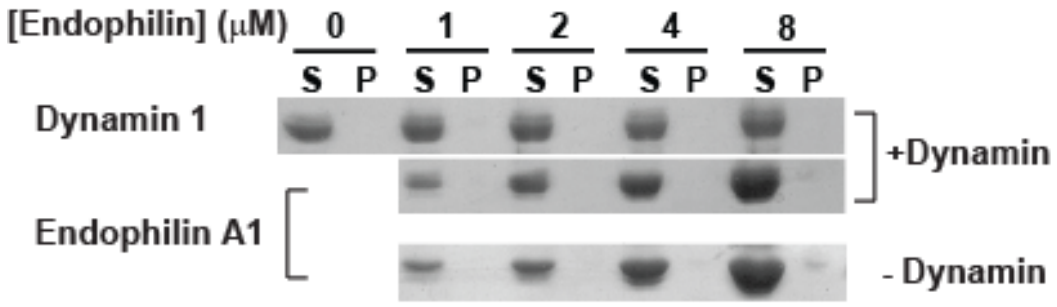

Figure S1. Sedimentation of various concentrations of endophilin A1 in the presence or absence of 1 μM dynamin 1. Conditions were identical to those used in Figure 2 in the main article.

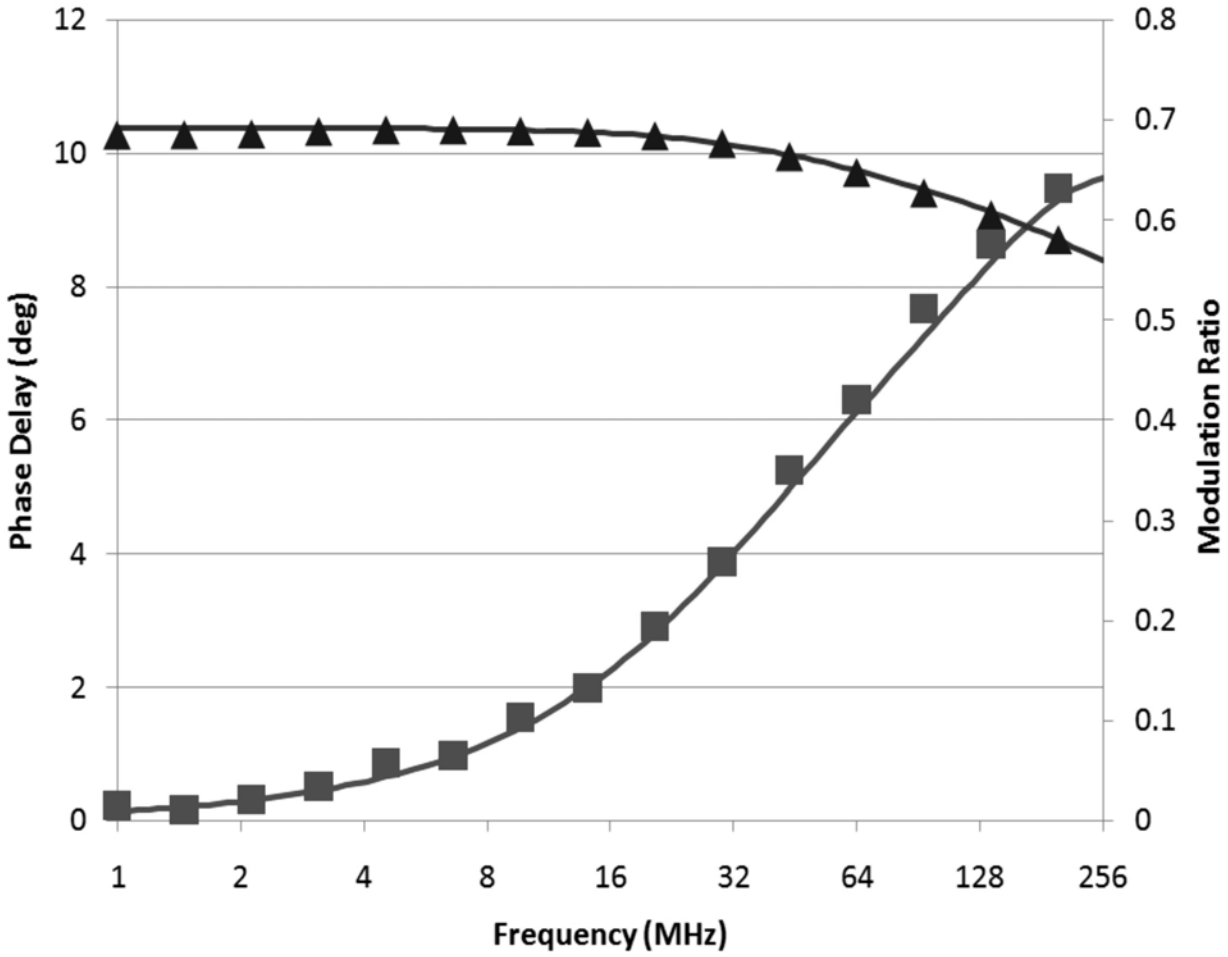

Figure S2. Dynamic polarization (modulation - black triangles and phase delay - black squares) of 24 μM endophilin labeled with Alexafluor 488. Fit parameters: τ_1 = 0.93 ns (10%), τ_2 = 3.62 ns (90%), ρ_1 =39.6 ns (0.13), ρ_1 =1.86 ns (0.15), where τ and ρ are the fluorescence lifetime and Debye rotational relaxation time, respectively.

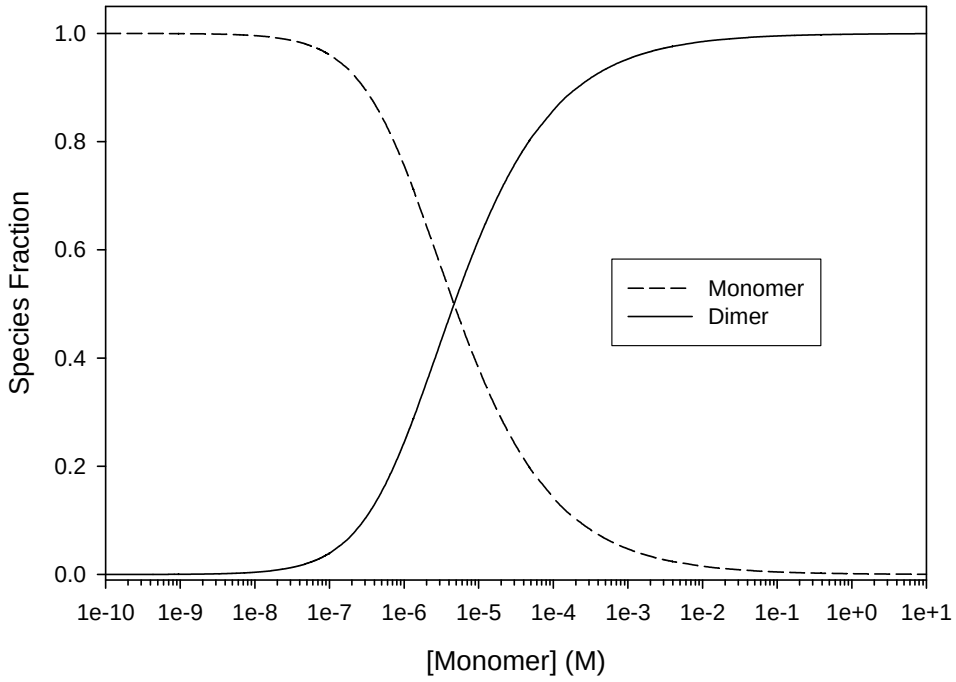

Figure S3. Species plot for endophilin assuming a monomer/dimer model and an equilibrium constant of ~5 μM. Sedimentation equilibrium runs were carried out on a Beckman XLI Analytical Ultracentrifuge at 4°C at two speeds (12 krpm and 19 krpm) and three endophilin concentrations. Concentrations were estimated as a function of radial position at 280 nm. Data were fit to various models with the best fit corresponding to a dimer/monomer equilibrium.

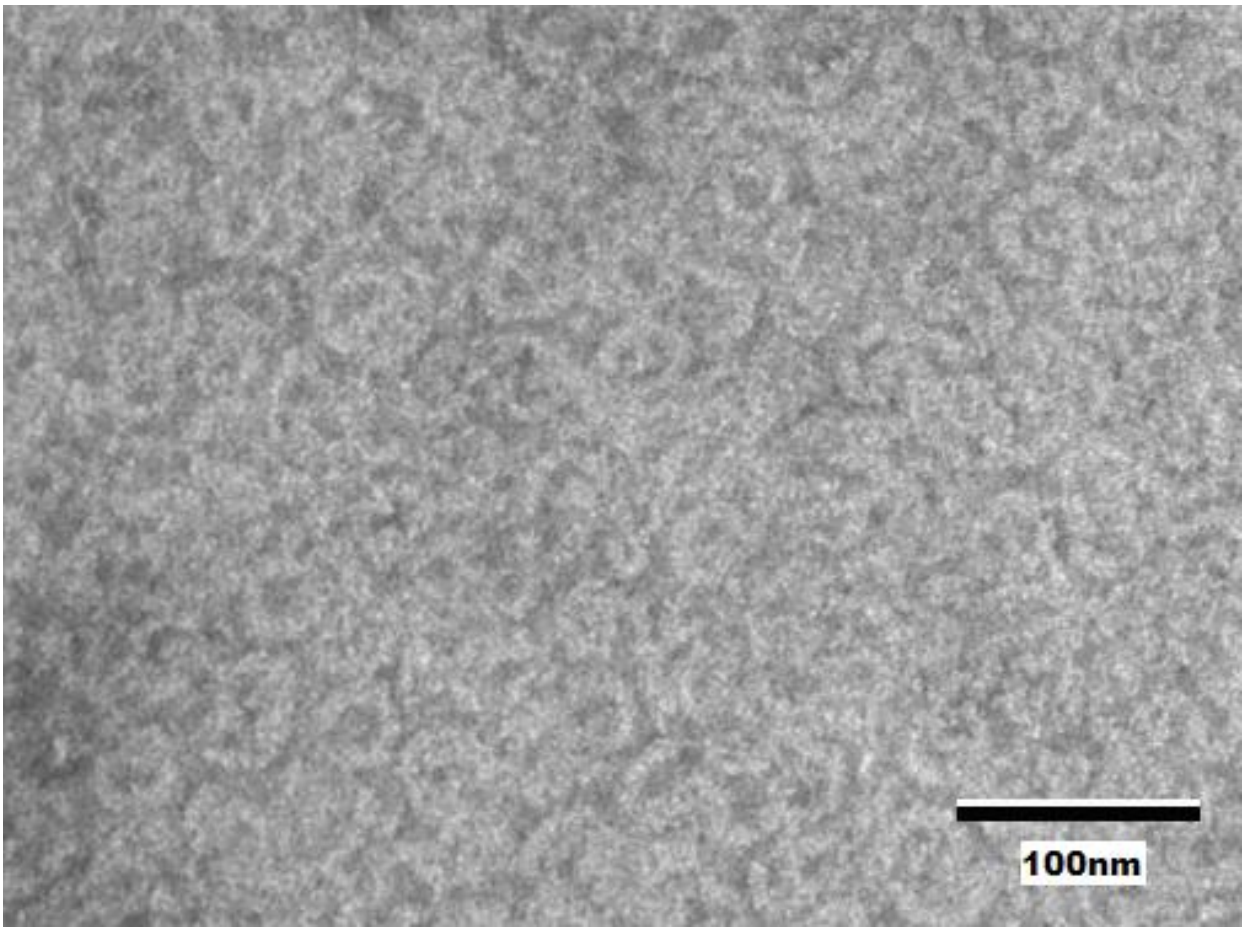

Figure S4. Negative stain TEM images of 1 μM dynamin 2 in the presence of 1μM endophilin A2 at 37°C approximately 90 seconds following dilution into 100mM NaCl buffer.

References:

1. Zacharias, D. A., J. D. Violin, A. C. Newton, and R. Y. Tsien. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science 296:913-916.