Supplemental Material and Methods

Recombinant DNA Constructs

The GST–cortactin fusion proteins 1–546, 491-546, 324-546, 1-326 and 1-81 were constructed by PCR amplification of the full length cortactin cDNA and subcloning of the resulting DNA in the BamHI and EcoRI sites of pGEX-6P1. All the constructs have been verified by sequencing.

In Vitro Protein Binding Assays.

GST-tagged cortactin or GST alone was mixed with His-tagged Hip1R in 200 µl of buffer B (20 mM Tris-Cl, pH 7.8, 100 mM KCl, 1 mM $MgCl_2$, 0.1% Tween 20), and incubated for 1 h at 4˚C. The reaction was added to 20 µl of glutathione sepharose and incubated for 1 h at 4˚C on a rotating wheel. Supernatants were collected and beads were washed 3 times with buffer B. Free and bound Hip1R were detected by Coomassie blue staining and western blotting using a polyclonal anti-Hip1R antibody (Engqvist-Goldstein et al., 1999) or an anti-His tag antibody (Novagen). Alternatively, GST-Hip1R domains immobilized on 20 µl glutathione sepharose were incubated with His-tagged cortactin and processed as above. Free and bound cortactin were detected by Coomassie blue staining and western blotting using a monoclonal anti-cortactin antibody (4F11 Upstate).

We also performed pull-down assays to measure the equilibrium dissociation constant. Increasing concentrations of GST-tagged cortactin bound to glutathione sepharose were incubated in the presence of Hip1R. The bound Hip1R was detected by western blotting

and quantified using Image J. The Hip1R bound fraction was plotted as a function of total GST-cortactin and the following equation was used to fit the data using Kaleidagraph. The fraction R of Hip1R bound to cortactin is $R = ([H0] + [C0] + K - (([H0] + [C0] + K)^2 - 4[H0] . [C0])^{0.5})/2 [H0]$, where, H0 is the concentration of Hip1R, C0 is the initial concentration of cortactin, and K is the equilibrium dissociation constant (K_d).

Co-Immunoprecipitations.

First, CCVs were purified from 100g of bovine brain (Pel-Freez Biologicals) as described in (Engqvist-Goldstein et al., 1999). Proteins were extracted using 0.5 M Tris MES pH 7.0, 1% Triton X100 and dialyzed in buffer A (20 mM HEPES-KOH, pH 7.2, 1 mM magnesium acetate, 150 mM potassium acetate). Anti-cortactin (4F11 Upstate) antibodies were then added to 200 µl of extract and incubated at 4˚C on a rotating platform. After 2 h, swollen protein G beads were added to the extract/antibody mixture (100 µl of 50% slurry) and incubated for an additional 2 h at 4˚C on a rotating wheel. The beads were washed three times with 0.5 ml of buffer A, loaded on SDS-PAGE gels, and analyzed by immunoblotting using a polyclonal anti-Hip1R antibody (Engqvist-Goldstein et al., 1999) and a monoclonal anti-cortactin antibody (4F11 Upstate).

Protein purifications.

Dynamin was purified as described in (Owen et al., 1998) and dialyzed overnight into 20 mM HEPES, pH 7.4, 500 mM NaCl, 4 mM DTT, 2mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 10 µg/ml benzamidine. Recombinant GST-His-tagged N-WASP (Rohatgi et al., 2000), His-tagged Hip1R and the truncated versions were purified from insect cells, and

the GST-tagged domains of Hip1R were purified from *E. coli* as described in (Engqvist-Goldstein et al., 2001), and dialyzed into 20 mM Tris pH 7.5, 100 mM KCl, 1mM DTT. Arp2/3 complex was purified from bovine brain as described in (Egile et al., 1999). Recombinant His-tagged cortactin was purified from *E. coli* as described in (Uruno et al., 2001).

References

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