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Supplemental materials and methods

Cell lines

Swiss 3T3 cells were obtained from ATCC. NIH3T3 fibroblasts were infected with pBabe-Hygro (Morgenstern and Land, 1990) or pBabe-Hygro-v-Src (Aftab et al., 1997) retroviruses packaged in ψ-2 cells and selected in 50 μg/ml Hygromycin. Resistant clones were isolated and screened for v-Src expression by immunoblotting with anti-Src (2–17) mAb. NIH3T3 fibroblasts expressing Y527FSrc were obtained from S. Courtneidge (Lock et al., 1998). NIH3T3(tsUP1) cells stably expressing the temperature-sensitive mutant of v-Src (*LA90)* were obtained from J. Brugge (Maroney et al., 1992). All cell lines were cultured in DME supplemented with 10% FCS without antibiotics in a humidified incubator at 37°C and 5% CO_2. Cells were passaged for a maximum of 4 wk.

Inhibitors

Y-27632 was a gift of the Welfide Corporation (Osaka, Japan) and used at 10 μM for 18–24 h.

DNA constructs

The pGEX-KG-TAT-C3 construct encoding a membrane permeable form of the Rho specific inhibitor C3 exoenzyme was obtained from C. Marshall (Sahai and Marshall, 2003). pcDNA3 plasmids with NH_2-terminal myc-tagged mutants of RhoA (Q63L and S19N) were gifts of R.G. Qiu (Qiu et al., 1995). pGEX-2T-TRBD plasmid encoding GST-RBD was a gift of M. Schwartz (Ren et al., 1999).

GST-RBD-AAA

To confirm that soluble GST-RBD specifically recognizes Rho[GTP] but not inactive Rho[GDP], we generated a mutant of the rhotekin RBD (RBD-AAA) containing three alanine substitutions (R37A, R39A, and D40A). These residues are analogous to those in the related Rho effector PKN that mediate contacts with Rho[GTP] (Maesaki et al., 1999). Mutant RBD (RBD-AAA) was generated by site-directed mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Arg37, Arg39, and Asp40 of mouse rhotekin were mutated to Ala using the primers: 5′-CTAGATCAT GAGATCGCGATGGCGGCTGGGGCCTGCAAGCTGCTG-3′, and 5′-CAGCAGCT-TGCAGGCCCCAGCCGCCATCGCGAT CTCATGATCTAG-3′. Mutations were confirmed by DNA sequencing, and RBD-AAA sub-cloned into BamH1/EcoRI sites in the original pGEX-2T vector. GST-RBD-AAA was a soluble protein and bound to Rho[GTP] in biochemical assays with reduced affinity.

GTPase pull-down assays

Biochemical affinity pull-down assays to measure Rho[GTP] were performed essentially as described previously (Ren and Schwartz, 2000). The binding efficiency of each batch of GST-RBD was titrated, and Rho pull-down assays were performed with 30–60 μg GST-RBD per sample. A small sample of each cleared lysate was reserved for analysis of total Rho protein present in the original sample.

Protein purification

GST, GST-RBD, and GST-RBD-AAA were purified as described previously (Ren and Schwartz, 2000). Of note, bacterial cultures were monitored by spectrometry and fusion protein expression was induced with 0.5 mM IPTG (final concentration) for 2 h at 30°C. All buffers were freshly made. All tubes, reagents, and rotors were prechilled on ice before use and all steps were performed in the cold room. GST-fusion proteins were purified in batch on glutathione-Sepharose 4B® beads (Amersham Biosciences). For in situ Rho[GTP] affinity assays, proteins were eluted from beads with three incubations of 20 min at 4°C in a total of three bed volumes of 10 mM reduced glutathione in a Tris buffer, pH 8.0. Soluble Tat-C3 was purified from BL21λDE3(pLysS) bacteria following a 3-h induction with 0.3 mM IPTG at 32°C. Bacterial lysates containing GST-Tat-C3 were incubated with glutathione-Sepharose 4B® beads for 2 h at 4°C. Beads were washed extensively, and the Tat-C3 was cleaved by incubation with thrombin (1 U/1 mg fusion protein) O/N at 4°C. After removal of soluble thrombin with p-aminobenzamidine agarose beads (Sigma-Aldrich), recombinant Tat-C3 was dialyzed 18–24 h in cold PBS. Recombinant Tat-C3 was tested for activity in vitro on cell lysates (Aktories et al., 1988) and in vivo on cells treated for 2–24 h. Activity was assessed by a mobility shift of Rho by ~1 kD in polyacrylamide gels, detected by immunoblot analysis. For experimental samples, purified soluble Tat-C3 dialyzed in PBS was added to culture medium (0.5 μM final concentration) for 18–24 h.

Antibodies and reagents

Anti-Src (2–17) mAb (Rigaudy et al., 1994) was purified from hybridoma supernatants. Anti-RhoA mAb (26C4), anti-Myc mAb (9E10), and polyclonal anti-GST antibody (Z-5) were obtained from Santa Cruz Biotechnology, Inc. Anti-RhoA/B/C mAb was obtained from Transduction Laboratories. Anti-GST mAb (26H1), anti-Myc-tag pAb and HRP-conjugated anti–

mouse and rabbit secondary antibodies were obtained from Cell Signaling Technologies. Anti-Fish pAb was a gift from S. Courtneidge (Lock et al., 1998). Anti-phosphotyrosine mAb (4G10) and anti-cortactin mAb (4F11) were purchased from Upstate Biotechnology. Most other reagents were purchased from Sigma-Aldrich. Gelatin Oregon green® 488 conjugate, anti–mouse IgG biotin conjugate; anti–rabbit, and anti–mouse IgG Alexa Fluor® 546 conjugate; streptavidin Alexa Fluor® 488 conjugate; rhodamine and Alexa Fluor® 488 phalloidin conjugates; and SlowFade® reagent were purchased from Molecular Probes.

Transient transfection, lysate preparation, and immunoblotting

Adherent cells were transfected with Lipofectamine Plus® (Invitrogen); transient transfection conditions were optimized for maximum expression and without significant toxicity to Src-transformed cells. For Rho[GTP] affinity assays, cells were processed 24 h after transfection. For immunoblot analysis, proteins were resolved by SDS-PAGE and transferred to Immobilon PVDF filters (Millipore). Blots were incubated 30 min to overnight in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk or 3% BSA. The blots were incubated with primary antibody overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies, and immunolabeled proteins were visualized with Western Lightning™ ECL chemicals (NEN Life Science Products).

Immunocytochemistry

Cells growing on glass coverslips were fixed in 4% PFA in PBS for 10 min, incubated 30 min in 3% BSA, 0.1 M glycine, 0.05% Triton X-100 in PBS, and 15 min in the same buffer containing 15% goat serum. Primary antibodies were incubated with samples overnight at 4°C. Cortactin and Fish antibodies were used at 1:500, 9E10 at 1:1,000, and myc-tag pAb at 1:800. Signals were developed using either Alexa Fluor® 546 goat anti–rabbit or anti–mouse IgG (1:200) for 45 min RT, or biotin anti–mouse or anti–rabbit IgG (1:200) for 45 min RT followed by a 30-min incubation with streptavidin Alexa Fluor® 488. F-actin was stained using rhodamine- or Alexa Fluor® 488-phalloidin (1:100) for 30 min. Samples were mounted in SlowFade® antifade reagent containing DAPI.

Tyramide Signal Amplification

Where indicated, the Tyramide Signal Amplification™ System (NEN Life Science Products) was used in the in situ Rho [GTP] detection assay. Fusion proteins were used at greatly reduced concentrations (10–20 μg/ml) to decrease potential nonspecific binding. Endogenous peroxidase activity was quenched 30 min in 3% hydrogen peroxide in PBS. After incubation with the corresponding fusion protein, samples were washed three times in washing buffer and incubated 30 min in the blocking buffer provided by the manufacturer. Anti-GST mAb was used at 1:20,000 and biotin anti–mouse IgG at 1:5,000. Fluorescein-Tyramide was incubated for 5 min and samples processed for fluorescence microscopy.

Imaging

Images were collected at RT using a 510 confocal laser scanning microscope (LSM; Carl Zeiss MicroImaging, Inc.) using 510 LSM software (v. 3.7; Carl Zeiss MicroImaging, Inc.) fitted with 40× 40 oil (NA 1.3; Carl Zeiss MicroImaging, Inc.) and 100× 100 oil (NA 1.4; Carl Zeiss MicroImaging, Inc.) objectives. DAPI was excited by a coherent enterprise laser using the 363-nm primary line; emissions from DAPI were detected using a band pass 385–470 filter before the photomultiplier tube. Alexa Fluor® 488 and Oregon green® 488 were excited by an Argon ion laser using the 488 nm primary line, and emissions were detected using a band pass 505–550 filter before the photomultiplier tube. Rhodamine and Alexa Fluor® 546 were excited by a Helium Neon laser using the 546-nm primary line, and emissions were detected by using a long pass 650 filter before the photomultiplier tube. Images were exported as TIFF files for subsequent processing with Adobe Photoshop® v.7.

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