Supplementary information

Materials and Methods

Yeast two-hybrid screening

A DNA fragment of the AMAP1 PRD (aa 704-1076), cloned into pBTM116, was used as a bait to screen a HeLa cDNA library (Clontech) with a two-hybrid system using *S. cerevisiae* L40 as a host. To examine the interaction with the AMAP2 PRD, a cDNA fragment encompassing the amino acids 761-950 cloned into pBTM116 was used.

Antibodies

Rabbit polyclonal antibodies against AMAP1 and AMAP2 were raised using GST-fused peptides corresponding to amino acids 935-1002 (AMAP1) and 871-929 (AMAP2), respectively. The antibodies were purified by ammonium sulphate precipitation followed by antigen-peptide affinity chromatography. The anti-GST antibodies were then removed by a GST affinity column. Other antibodies were as follows: anti-cortactin, anti-GST (Upstate), anti-paxillin (Transduction Laboratories), anti-β-actin (Sigma-Aldrich), anti-HA (Covance Research Products), anti-Xpress (Invitrogen), Cy2-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories). IgG1 from non-immune mice was purchased from Sigma-Aldrich.

Peptide binding

Proline-rich peptides were synthesized on cellulose membranes using SPOTs KIT (Genosys) according to the manufacturer's instructions. After blocking, membranes were hybridized with 0.1 μg/ml of ^{32}P-labeled GST proteins at 4°C overnight, then washed three times with Hyb 75 buffer (20m M HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40) (Hashimoto *et al,* 2004a) containing 5% BSA. Radioactivities retained on filters were measured using FLA-2000 and MacBAS Ver. 2.5 (Fujifilm). GST-cortactin SH3 or GST (a product of pGEX-2TK) was phosphorylated using the catalytic subunit of cAMP-dependent kinase

(Sigma-Aldrich) and [γ^{32}P]ATP, according to the manufacturer's instructions. The specific radioactivity of the probes was about $5x10^6$ cpm/μg.

Cellular extract preparation, protein binding, immunoprecipitation, and immunoblot analysis

Cell lysates were prepared with 1% NP-40 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin and 3 μg/ml pepstatin A). *In vivo* protein binding assays were performed by precipitating GST-fusion proteins from 500 μg of cell lysate using glutathione-beads (Amersham Biosciences). For the *in vitro* protein binding assay, 5 μg of GST-fusion proteins, expressed in bacteria and purified on glutathione-beads, were incubated with 300 μg of cDNA-transfected cell lysates. For coprecipitation analysis of paxillin and AMAP1 with cortactin, 500 μg of cell lysate was prepared as described previously (Bowden *et al,* 1999), and used for immunoprecipitation with anti-cortactin coupled with anti-mouse IgG-Sepharose beads (Sigma-Aldrich). Proteins precipitated were then subjected to immunoblotting analysis after being separated by SDS-PAGE, as described previously (Hashimoto *et al,* 2004a), and detected using an enzyme-linked chemiluminescence method (Amersham Biosciences). Amounts of proteins were then measured by a densitometer (GT-X700 Scanner, Epson) using ImageJ version 1.33 (NIH) software, and data were presented as the mean values together with SEM from at least three independent experiments. When necessary, 5 μg of total cell lysate was included.

Real-time PCR

Total cellular RNA was isolated using Trizol (Life Science). Random-primed cDNAs, prepared from 2 μg of cellular RNAs using the SuperScript first-strand cDNA synthesis kit (Invitrogen), were subjected to real-time PCR amplification analysis using the LightCycler FastStart DNA Mater SYBR Green I (Roche) and the LightCycler (Roche), according to the manufacturer's instructions.

Primers used were 5'- GTATAGAATGTTCTGGCATC-3' and 5'-CTGATGAAGTTGAACAGGTC-3' for AMAP1. Human β-actin primers were from Takara (Kyoto, Japan). PCR amplification conditions were 95 ºC for 10 min followed by 40 cycles (95 ºC for 15 sec, 56 ºC for 5 sec, 72 ºC for 10 sec) for AMAP1, and by 40

cycles (95 ºC for 15 sec, 58 ºC for 5 sec, 72 ºC for 10 sec) for β-actin.

Immunofluorescent microscopy

Immunofluorescent analysis was done as described previously (Hashimoto *et al*, 2004b). Briefly, cells were fixed in 4 % paraformaldehyde in PBS for 10 min at room temperature, 16 h after replating onto collagen unless otherwise indicated. After permeabilization with 0.1% Triton X-100 in PBS for 10 min at room temperature, cells were blocked with 5% BSA in PBS. Cortactin was visualized using an anti-cortactin antibody coupled with a Cy2-conjugated antibody against mouse IgG (Jackson Immuno Research Laboratories), F-actin was visualized with Texas Red-conjugated phalloidin (Molecular Probes) and AMAP1 was visualized with an anti-AMAP1 antibody coupled with a Cy5-conjugated antibody against rabbit IgG (Jackson Immuno Research Laboratories). Fluorescence microscopy was performed using a confocal laser-scanning microscope (LSM 510, Carl Zeiss) and the attached software. Representative figures from analyzing more than 50 cells are shown.

Peptide injection

$1x10^5$ cells were seeded onto glass-bottomed dishes (MatTek), which were coated with fluorescence-conjugated, cross-linked gelatin films, in serum-free growth media. Twelve h later, cells were microinjected using a microinjection system (Eppendorf) with 1 mg/ml of the 4th proline-rich peptide of AMAP1 (SKKRPPPPPPGHKRT) or a proline-rich peptide of AMAP2 (AMVLQPPAPMPRKSQ), together with 1mg/ml Alexa Fluor 488-conjugated dextran (Molecular Probes), and recovered for 2 h at 37˚C in a CO_2 incubator. After media were exchanged to the serum-containing growth media, cells were incubated for a further 12 h before being subjected to invadopodia formation analysis.

References

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