Junctional protein MAGI-3 interacts with receptor tyrosine phosphatase β (RPTPβ) and tyrosine-phosphorylated proteins

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Accepted 2 December 2002

Journal of Cell Science 116, 1279-1289 © 2003 The Company of Biologists Ltd

doi:10.1242/jcs.00302

Summary

Receptor protein tyrosine phosphatase β (RPTPβ) mediates cell-cell and cell-matrix interactions. By searching for intracellular proteins that interact with the cytoplasmic region of this phosphatase using the two-hybrid method, we identified several proteins containing PDZ domains. One of these proteins, MAGI-3, contains a guanylate-kinase-like region, six PDZ and two WW domains. The interaction between RPTPβ and MAGI-3 was confirmed by co-immunoprecipitation and pulldown experiments in transfected cells. Immunofluorescence and immunoelectron microscopy revealed that MAGI-3 is concentrated in specific sites at the plasma membrane and in the nucleus. In epithelial cells, MAGI-3 was localized with ZO-1 and cingulin at tight junctions, whereas in primary cultured astrocytes it was found in E-cadherin-based cell-cell contacts and in focal adhesion sites. Although MAGI-3 itself was not phosphorylated on tyrosine residues, it became associated with tyrosine-phosphorylated proteins following a short treatment of the cells with vanadate. In glioblastoma SF763T cells MAGI-3 was associated with a tyrosine-phosphorylated protein with the apparent molecular weight of 130 kDa, whereas in Caco2 cells it was associated with a 90 kDa protein. Finally, we show that p130 served as a substrate for RPTPβ and that its dephosphorylation required the C-terminal sequence of the phosphatase, which mediated the interaction with MAGI-3. These findings suggest a possible role for MAGI-3 as a scaffolding molecule that links receptor tyrosine phosphatase with its substrates at the plasma membrane.

Key words: PDZ domain, Tyrosine phosphatase, RPTPβ, Tight junctions, Adherens junctions, MAGI

Introduction

Receptor protein tyrosine phosphatase β (RPTPβ, also known as RPTPζ) and RPTPγ are members of a distinct group of phosphatases, characterized by the presence of carbonic-anhydrase-like (CAH) and fibronectin type III (FNIII) domains in their extracellular region (Barnea et al., 1993; Krueger and Saito, 1992; Levy et al., 1993). In common with other receptor protein tyrosine phosphatases, RPTPβ mediates cell-cell and cell-matrix interactions and is implicated in cell adhesion and cell migration (Adamsky et al., 2001; Grumet, 1993). RPTPβ exists as three isoforms: short and long receptor forms, and a third secreted protein containing the extracellular domain of the long receptor form that is produced as a chondroitin sulfate proteoglycan (Barnea et al., 1994; Maurel et al., 1994). The three forms of RPTPβ differentially bind to multiple ligands, including neuronal recognition molecules (Milev et al., 1994; Milev et al., 1996; Peles et al., 1995), extracellular matrix components (Adamsky et al., 2001; Barnea et al., 1994; Grumet et al., 1994; Milev et al., 1997; Xiao et al., 1997) and heparin-binding growth factors (Maeda et al., 1999; Maeda et al., 1996; Meng et al., 2000; Milev et al., 1998). Although these studies indicate a role for RPTPβ in cell communication, little is known about the role of proteins that interact with the intracellular region of this phosphatase. RPTPβ interacts with four proteins that have a role in cytoskeletal changes within the cell. These include the postsynaptic density protein PSD95 (Kawachi et al., 1999), β-catenin, a major constituent of the cadherins-based adherens junctions (Meng et al., 2000), the α and β subunits of Na^+ channels (Ratcliffe et al., 2000) and GIT1, a protein involved in the regulation of small GTP-binding proteins CDC42 and Rac (Kawachi et al., 2001).

Proteins containing PDZ (PSD-95/discs large/zona occludens 1) domains have been identified as key components in the organization of protein complexes at the plasma membrane (Fanning and Anderson, 1999). These proteins have been implicated in several functions, including localization of proteins to specialized cell junctions (Gonzalez-Mariscal et al., 2000), clustering of transmembrane receptors (Sheng and Pak, 2000) and the recruitment of cytosolic proteins to generate multi-signaling complexes (Scott and Zuker, 1998). Many PDZ-domain-containing proteins are found in a multidomain arrangement that also includes other modules of protein-protein interactions. One particular class consists of the MAGUKs (membrane-associated-guanylate kinase), a family of scaffolding proteins containing several PDZ domains, SH3 domains and a catalytically inactive guanylate kinase domain, all of which mediate protein-protein interactions (Harris and Lim, 2001). The presence of three distinct domains that

mediate protein-protein interaction, along with the ability of these domains to form higher-order oligomers through intermolecular interactions between the SH3 and the guanylate kinase domain, enables the MAGUKs to recruit signaling proteins and to control the assembly of multivalent protein complexes (McGee et al., 2001; Nix et al., 2000). MAGI (membrane-associated guanylate kinase with inverted orientation) proteins are a distinct subgroup of the MAGUKs and include MAGI-1/BAP1 (Dobrosotskaya et al., 1997; Shiratsuchi et al., 1998), MAGI-2/AIP/ARIP/S-SCAM (Hirao et al., 1998; Shoji et al., 2000; Wood et al., 1998) and MAGI-3 (Wu et al., 2000b). In the present study we have identified the rat homologue of MAGI-3 as a protein that interacts with the C-terminal tail of RPTPβ. Furthermore, we found that MAGI-3 interacts with tyrosine-phosphorylated proteins in different cell types, suggesting that it may serve as a scaffold to position substrates for receptor tyrosine phosphatases at the plasma membrane.

Materials and Methods

Yeast two-hybrid system

The cytoplasmic region of RPTPβ (amino acids 798-1440, according to the short receptor form of RPTPβ) was cloned in frame with the binding domain of lexA in pBTM116 and was used as a bait to screen a SF763T glioblastoma cDNA library cloned in pGAD-GH (Clontech). Yeast transformation of L40 strain, screening for clones grown in plates lacking leucine, tryptophan and histidine, as well as plasmid rescue were done as previously described (Vojtek et al., 1993). Other pBTM constructs used for the two-hybrid analysis include the first phosphatase domain of RPTPβ (D1, amino acids 802-1163), the second phosphatase domain (D2, amino acids 1118-1440), the RPTPβ C-terminal tail (CT, amino acids 1382-1440) and the entire cytoplasmic region of RPTPβ lacking its last five amino acids (D12dCT amino acids 798-1435).

cDNA cloning and constructs

For cloning of a full-length MAGI-3, an *Eco*RI-*Xho*I fragment containing the third and fourth PDZ domains, isolated from one of the clones obtained from the two-hybrid library (pGH#707), was used as a probe to screen a ZAP-PC12 cDNA library. The sequence of rat *Slipr/MAGI-3* cDNA was determined on both strands by priming with synthetic oligonucleotides and deposited in GenBank (accession number AF255614). Expression constructs were made by subcloning the full-length cDNA into pCDNA3 (Invitrogen) to generate pC3-rMAGI-3. The RPTPβ expression construct containing a hemagglutinin tag has previously been described (Adamsky et al., 2001).

Fusion proteins and antibodies

GST-fusion proteins containing the intracellular region of RPTPβ (GST-βD12, amino acids 799-1440), the first phosphatase domain (GST-βD1, amino acids 799-1154), the second phosphatase domain including the C-terminal tail (GST-βD2, amino acids 1120-1440) and the intracellular region of RPTPβ lacking the last eight amino acids (GST-βD12dCT) were made by cloning the corresponding cDNA fragments into pGEX-6P (Pharmacia-Amersham). A catalytically inactive RPTPβ mutant was constructed by replacing aspartic acid (1026) with alanine residues to generate GST-βD12DA. Polyclonal antibodies against MAGI-3 were generated by immunizing rabbits with a synthetic peptide (RLNRTELPTRSAPQES, corresponding to amino acid residues 835-850 of MAGI-3) coupled to keyhole limpet hemocyanin (Sigma). Antibodies were affinity purified on a column of the peptide antigens covalently coupled to agarose beads (Pierce). A second antibody to MAGI-3 was generated by immunizing rabbits with a GST-fusion protein containing the third and fourth PDZ domains of rat MAGI-3 (Ab#M23). Affinity purification was performed by first removing the antibodies against GST using Sepharose-GST (Pierce) and then running it on a column of GST-MAGI-3#707. Antibodies to HA-tag were purchased from Roche Molecular Biochemicals and antibodies to ZO-1 and phosphotyrosine (PY20) from Transduction Laboratories. Antibodies against β-catenin, desmoplakin and cingulin were generously provided by Benny Geiger (The Weizmann Institute).

Northern blots and RT PCR

A 1 kb DNA fragment corresponding to positions 1404-2427 of rat MAGI-3 cDNA was used as a probe for northern blot analysis. The DNA fragment was isolated, labeled by random priming, and purified on a Sepharose G-50 column (Amersham-Pharmacia). Hybridization to multiple tissue northern blots (MTN Blots, Clontech) was carried out as described previously (Poliak et al., 1999). PCR analysis was done with various cDNA sources using a set of primers (5-GGCAAAGTCATAAATAAAG-3, 5-CTCTGCAAGAAAGCC-3) specific to MAGI-3, which did not recognize rat MAGI-1 or MAGI-2.

Immunoprecipitation and peptide pulldown experiments

Immunoprecipitation and immunoblotting analyses from different cell lines or rat brain membrane lysates were done essentially as described previously (Adamsky et al., 2001). Co-immunoprecipitation from HEK-293T cells transfected with RPTPβ, MAGI-3, or both proteins, was done using a solubilization buffer containing 1% Triton X-100 as previously described (Peles et al., 1995). For GST-pulldown experiments, SF763T cells were solubilized in TNTG buffer (20 mM Tris 7.5, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol and protease inhibitors), and the lysates were incubated with various GST-fusion proteins coupled to glutathione-Sepharose (Amersham-Pharmacia). For peptide-pulldown experiments, rat brains were solubilized in RIPA buffer (10 mM sodium phosphate pH 7, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 1 mM PMSF), the lysates were diluted 1:1 with TNTG, and incubated with 20 μg of biotinylated peptides coupled to Neutravidin beads (Pierce). Bound proteins were washed three times with TNTG, separated on SDS gels and immunoblotted with an anti-MAGI-3 antibody. Peptide used for pulldown experiments include βCT-NIAESLESLV, βCTS-SISENEVLAL, Caspr2CT-NFTETIDESKKEWLI and Caspr2dCT-NFTETIDESKKE (Poliak et al., 1999). For co-immunoprecipitation of phosphorylated proteins, cells were treated with 1 mM Na_3VO_4 for 30 minutes at 37°C and solubilized using RIPA buffer containing 1mM Na_3VO_4. Lysates were immunoprecipitated with anti-MAGI-3 antibodies (BM#4112; Sigma), and were western blotted with Py-20 anti-phosphotyrosine antibodies (Transduction Laboratories).

Dephosphorylation experiments

Phosphorylated p130 was co-precipitated with an anti MAGI-3 antibody from vanadate-treated SF763T cells. The immunocomplexes where divided equally between several tubes and incubated with GST-RPTPβ fusion protein coupled to beads in 50 μl of buffer containing 25 mM MES, pH 5.5, 5 mM DTT or with 5U shrimp alkaline phosphatase (Roach) in 50 mM Tris (pH 8.0), 5 mM DTT. All reactions were carried out for 2 hours at 23°C with gentle shaking. Dephosphorylation of p130 was then monitored by immunoblotting using anti-PY antibodies (PY20). Phosphatase activity of GST-RPTPβ constructs was measured as *p*-phosphate hydrolysis in 0.5 ml of 25 mM MES, pH 5.5, containing 5 mM DTT and 10 mM *p*NPP incubated

at 23°C. The reaction was stopped by the addition of 100 μl of 1 N NaOH, and the absorbance was measured at 405 nm.

Immunofluorescence

Caco2 cells or cultures of primary rat astrocytes (Adamsky et al., 2001) were grown on glass coverslips precoated with 10 μg/ml polylysine. Slides were fixed/permeabilized with 0.2% Triton X-100/4% paraformaldehyde for 3 minutes and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 minutes. Primary antibodies were diluted in 0.1 M PBS, pH 7.4, containing 0.1% Triton X-100 (PBST) and incubated for 1 hour. After three washes with PBS, slides were incubated with Alexa-488-conjugated anti-mouse and Cy3-conjugated anti-rabbit antibodies in DAPI-containing PBTS. Slides were subsequently washed with PBS and mounted with Elvanol for observation. Immunofluorescence slides were viewed and analyzed using a BioRad confocal microscope or a Zeiss Axioplan microscope equipped with a SPOT-II (Diagnostic Instruments) cooled CCD camera.

Immunoelectron microscopy

For immunogold electron microscopy, Caco2 cells were fixed with a freshly prepared solution of 4% paraformaldehyde, 2% acrolein and 0.1 M sucrose in 0.1 M cacodylate buffer containing 5 mM $CaCl_2$ for 1 hour at 24°C. The cells were scraped from the culture dish; the pellets were incubated in 10% gelatin at 37°C for 30 minutes and postfixed in the same fixative at 40°C for 24 hours. Fixed cell pellets were cryoprotected overnight with 2.3 M sucrose in cacodylate buffer and frozen by injection into liquid nitrogen. Ultrathin (75 nm) sections were cut with a diamond knife (Drukker) at –115°C using a Reichert Ultracat-S ultramicrotome. The sections were transferred to 200-mesh nickel grids coated with formvar. The sections were treated with PBS-containing 0.5% BSA, 3% normal goat serum (Sigma), 0.1% glycine and 1% Tween 20 for 5 minutes to block non-specific binding, followed by 2 hours of incubation with M-23 antibodies. After extensive washing in PBS-0.1% glycine, the primary antibody was detected with goat anti-rabbit 10 nm colloidal gold conjugate (1:20; Zymed). The grids were then washed in PBS-glycine, stained with neutral uranyl acetate oxalate for 5 minutes, quickly washed and then stained with 2% uranyl acetate in H_2O for 10 minutes. Embedding was done in 2% methyl cellulose/uranyl acetate.

Results

Isolation of MAGI-3, a scaffolding protein that interacts with RPTPβ

In order to isolate proteins that interact with RPTPβ, we used its cytoplasmic domain as a bait to screen a glioblastoma cDNA library using the yeast two-hybrid method. This approach yielded 81 clones, which could be subdivided into two categories. The first consisted of the D2 domain of several protein tyrosine phosphatases, including PTPμ, PTPα and RPTPβ itself. The second group of proteins that interacted with RPTPβ contained proteins with PDZ domains, including PSD95 (Cho et al., 1992), Chapsyn-110 (Kim et al., 1996), MUPP1 (Ullmer et al., 1998; Poliak et al., 2002) and a novel sequence similar to the third and fourth PDZ domains of S-SCAM/MAGI-2 (Hirao et al., 2000). A DNA fragment of the latter obtained in the two-hybrid screen was used to isolate the full-length cDNA from the PC12 cDNA library. As shown in Fig. 1, this novel cDNA, which we termed *Slipr* (scaffolding like protein; GenBank accession number AF255614), has an open reading frame that encodes for 1179 amino acids and contains six PDZ, one guanylate kinase and two WW domains. The domain

A.

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   1  MSKTLKKKKH WLSKVQECAV SWAGPPGDLG AEIRGGAERG EFPVLGRLRD
  51  EPGGGGGTCC VVSGKAPSPG DVLLEVNGTP VSGLTNRDTL AVIRHFREPI
 101  RLKTVKPGKV INKDLRHVLS LQFQKGSIDH KLQQVIRDNL YLITIPCTTR
 151  APRDGEVPGV DVNFISVEQF KALEESGALL ESGTVDGNFV GTPKPPAEPS
 201  PFQPDPVDQV LFDNEFDTES QRKRTTSVSK MERMDSSLPE EEEDEDKEAV
 251  NGSGSMETRE MHSESSDCWM KTVPSVNQTN RSMDFRNVMM RDENLEPLPK
 301  NWEMAYTDTG TIVFIDHNTK TTTWLDPRLC KKAKAPEDCE DGELPVGWEK
 351  IEDPQVGTVV VDHLNQKTQF ENPVEEAKRK KQIGQAETHS AKTDVERAHF
 401  TRDPSQLKGV LVRASLKKST MGFGFTIIGG DRPDEFLQVK NVLKDGPAAQ
 451  DGKMAPGDVI VDINGNCVLG HTHADVVQMF QLVPVNQVVN LTLCRGVALP
 501  DDSEDPVVDI VAATPVINGQ SLAKGEACMS TQDFKLGAMV LDQNGKSGKL
 551  LSSDRLNGPS DSNEQRASLA SSGSSQPELV TIPLVKGPKG FGFAIADSPT
 601  GQKVKMILDS QWCQGLQKGD IIKEIVHQNV QNLTHLQVVE VLKQFPVGAD
 651  VPLLILRGGP CSPTKTAKMK TDTKETSGSL ETINEPTPQP MPFPPSIIRS
 701  PSPKLDPSEV VLKSKTLVED KPPNTKDLDV FLRKQESGFG FRVLGGDGPD
 751  QSIVIGAIIP LGAAEKDGRL RAADELMCID GIPVKGKSHK QVLDLMTTAA
 801  RNGHVLLTVR RKIFVGEKQP EDESPQAFSQ SGSPRLNRTE LPTRSAPQES
 851  VDVILQRKEN EGFGFVILTS KSKPPPGVIP HKIGRVIDGS PADRCGRLKV
 901  GDHISAVNGQ SIVDLSHDNI VQLIKDAGVT VTLTVVAEEE HHGPPSGTNS
 951  ARQSPALQHR PMGQAQATHI PGDRTALEGE VGKDVCSSYR HSWSDHKHLA
1001  QPDTAVISVV GSRHSQSLGC VPVELERGPR GFGFSLRGGK EVNMGLFILR
1051  LAEDGPAIKD GRIHVGDQIV EINGEPTQGI THTRAIELIQ AGGNKVLLLL
1101  RPGTGLIPDH GDWDIVSPSS SNVIVDEQPP PLPSSHSAAT FEESHVPVTE
1151  DSLIRVQTCE KAEELKDTVQ EKHFKWKPA
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B.

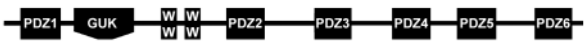

Fig. 1. Amino-acid sequences and domain organization MAGI-3. (A) Amino-acid sequence of rat MAGI-3. (B) Schematic organization. MAGI-3 contains six PDZ domains, as well as two WW domains and a guanylate kinase domain (GUK), which are located between the first and second PDZ domains. This organization is similar for all members of the MAGI family and is distinct from the original MAGUK proteins that contain an SH3 domain instead of the WW domains and in which the GUK domain is located at the C-terminal end of the protein.

organization of the encoded polypeptide is very similar to that of MAGI-1/BAP1 (Dobrosotskaya et al., 1997; Shiratsuchi et al., 1998), MAGI-2/AIP/ARIP/S-SCAM (Hirao et al., 1998; Shoji et al., 2000; Wood et al., 1998) and MAGI-3 (Wu et al., 2000b), and most probably represents the rat orthologue of MAGI-3. This cDNA was therefore redesignated rat *MAGI-3*.

Expression of MAGI-3 mRNA and protein

Analysis of the expression of MAGI-3 mRNA in various rat tissues by northern blots revealed the presence of a single 6.4 kb transcript in the brain, heart, lung, liver and at a lower level in the kidney (Fig. 2A). No MAGI-3 transcript was detected in the spleen, skeletal muscle and testis. The size of the transcript, as well as its expression in various tissues, differed from those of MAGI-1 (Dobrosotskaya et al., 1997; Shiratsuchi et al., 1998) and MAGI-2 (Hirao et al., 1998; Shoji et al., 2000; Wood et al., 1998), indicating that the probe we used specifically detected MAGI-3 and not the related MAGIs. RT-PCR analysis using a set of primers specific to *MAGI-3* detected its expression in rat brain, retina, astrocytes and in neuroendocrine GH3 cell lines but not in neural stem cells or testis (Fig. 2B). We next examined the expression of MAGI-3 protein in various cell lines, rat brain lysates and primary astrocytes using antibodies that were raised against a peptide that is found in MAGI-3 but not in MAGI-1

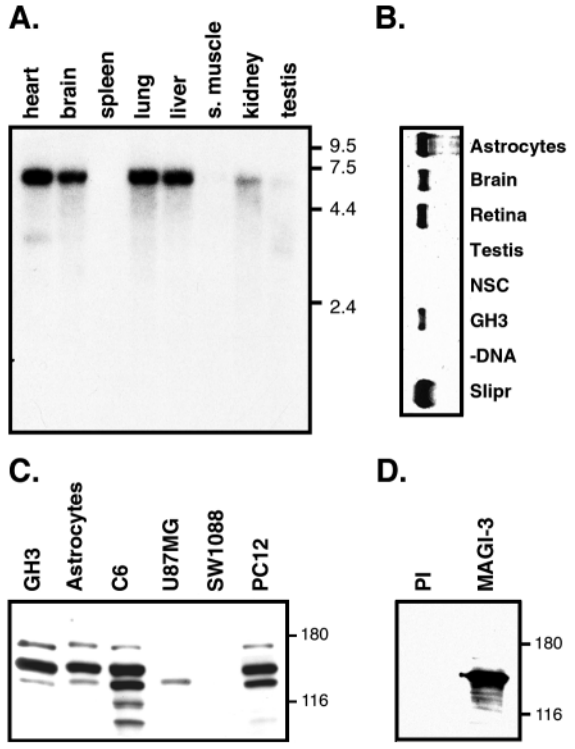

Fig. 2. Expression of MAGI-3 mRNA and protein. (A) A northern blot containing mRNA from various rat tissues hybridized with a DNA fragment containing the third and fourth PDZ domains of MAGI-3 as a probe. The autoradiogram is shown along with the location of molecular weight markers in kb. A single 6.4 kb transcript was detected in the indicated tissues. (B) RT-PCR analyses peformed with MAGI-3-specific primers on the indicated rat tissues, GH3 neuroendocrine cell line, neuronal stem cells (NSC) and primary astrocytes. MAGI-3 cDNA (MAGI-3) and a reaction containing no DNA (–DNA) were used as positive and negative controls, respectively. (C) Expression of MAGI-3 protein in various cell lines. Cell lysates made from the indicated cell lines or primary astrocytes were subjected to immunoprecipitation and western blot analysis using an antibody to MAGI-3. Molecular weight markers in kDa are shown on the right. Note that the lower two bands shown in C6 are degradation products of MAGI-3, which were not detected in other experiments. (D) Rat brain membrane lysates were immunoprecipitated with specific antibodies to MAGI-3 or with the preimmune serum (PI) as indicated, followed by immunoblotting with anti-MAGI-3 antibody.

and MAGI-2. As shown in Fig. 2C,D, this antibody, but not the pre-immune serum, recognized a major band at 140 kDa and two minor bands at 170 kDa and 130 kDa in GH3 cells, primary cultured astrocytes and in rat brain. In C6 and PC12 cells, two major forms at 140 kDa and 130 kDa, as well as a minor band at 170 kDa, were detected. Similar results were obtained using a monoclonal antibody specific to MAGI-3 (data not shown), suggesting that MAGI-3 has multiple isoforms, as do MAGI-1 and MAGI-2, which are generated by alternative splicing (Dobrosotskaya et al., 1997; Hirao et al., 2000). Nevertheless, although several splice isoforms of MAGI-3 do exist in the databases (data not shown), we found no indication that the minor 170 kDa band results from alternative splicing.

Association of RPTPβ with MAGI-3 is mediated by its C-terminal tail

To further corroborate the two-hybrid data, we examined whether RPTPβ interacts with MAGI-3 in transfected cells. As

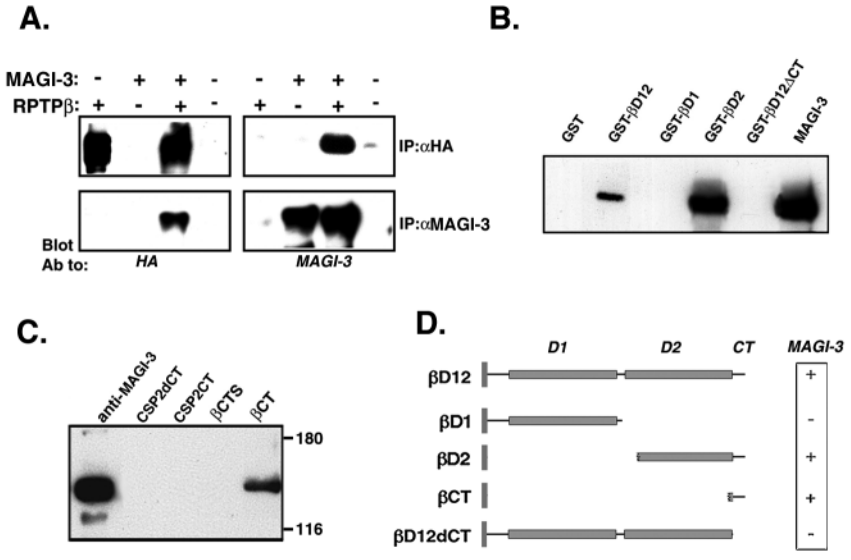

Fig. 3. Association of MAGI-3 with RPTPβ. (A) HEK-293T cells were transfected with MAGI-3, HA-tagged RPTPβ or with both MAGI-3 and RPTPβ as indicated (–/+). Cell lysates were subjected to immunoprecipitation with an antibody against the HA-tag that recognizes the extracellular domain of RPTPβ (IP:αHA) or an antibody to MAGI-3 (IP:αMAGI-3) as indicated on the right. Washed immunocomplexes were separated on an SDS gel and immunoblotted with an antibody to HA-tag (left panels) or to MAGI-3 (right panels). (B) Pulldown of MAGI-3 by the cytoplasmic domain of RPTPβ. Lysates of HEK-293T cells expressing MAGI-3 were mixed with agarose-bound GST or GST-fusion proteins containing the entire cytoplasmic domain of RPTPβ (βD12), the first phosphatase domain (βD1), the second phosphatase domain (βD2) or a mutant form lacking the C-terminus (βD12dCT) as indicated. Bound proteins were immunoblotted with an antibody to MAGI-3. Immunoprecipitation with an antibody to MAGI-3 (MAGI-3) was used as a control. (C) Interaction of the C-terminal tail of RPTPβ with MAGI-3. Rat brain membrane lysate was mixed with immobilized peptides corresponding to the C-terminal tails of RPTPβ (RPTPβCT), a peptide containing a scrambled sequence (βCTS), the C-terminal peptide of Caspr2 (CSP2CT) or a similar peptide lacking the last amino acid (CSP2dCT). Bound proteins were separated on SDS gels and blotted with an antibody to MAGI-3. As a positive control, a lysate sample used in the pulldown experiment was immunoprecipitated with an antibody to MAGI-3 (MAGI-3). Molecular weight markers are shown in kDa on the right. (D) Two-hybrid analysis. The ability of the different domains of RPTPβ to interact with a MAGI-3 construct, containing its third and a fourth PDZ domains, was examined using the two-hybrid method (–/+). All RPTPβ constructs containing the C-terminal tail interact with MAGI-3, unlike those that lacked this region.

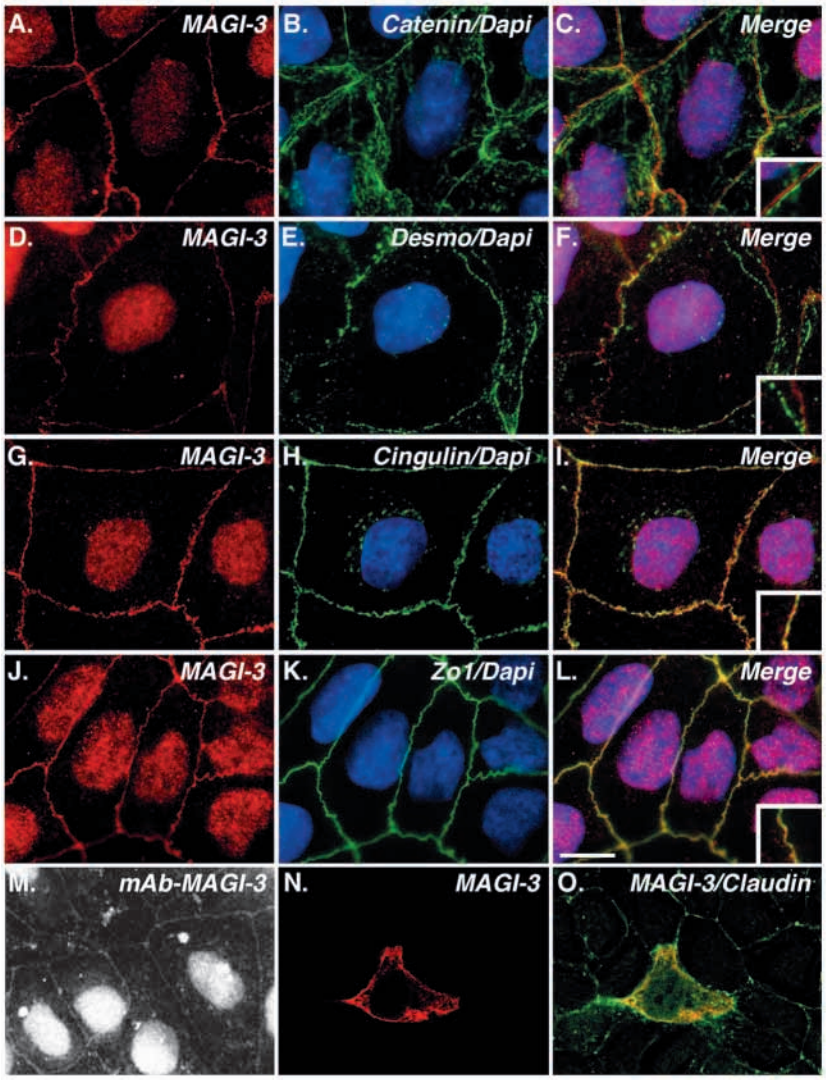

Fig. 4. Subcellular distribution of MAGI-3 in epithelial cells. Caco2 colon carcinoma cells were double labeled using a polyclonal antibody to MAGI-3 (A,D,G,J) and monoclonal antibodies to β-catenin (B), desmoplakin (E), cingulin (H) or ZO-1 (K) as indicated. MAGI-3 is shown in red, all other proteins in green, and DAPI-labeled nuclei are blue. Merge images of each set are shown on the right. Higher magnification of the region labeled with an asterisk from the merge image is shown in the inset of each panel. Note that MAGI-3 colocalized with cingulin and ZO-1 but not with β-catenin or desmoplakin. Similar cellular distribution of MAGI-3 in Caco2 cells was also detected using a distinct monoclonal antibody (M). The localization of MAGI-3 in transfected MDCK cells, which do not express endogenous MAGI-3, is presented in panels N, or as a double staining with Claudin-1 (O; green). Bars, A-L, 15 μm; M, 20 μm; N-O, 40 μm.

shown in Fig. 3A, RPTPβ could be immunoprecipitated using an antibody against MAGI-3 from HEK-293T cells expressing both proteins but not from cells expressing MAGI-3 or RPTPβ alone, or from untransfected cells. Similarly, MAGI-3 was co-immunoprecipitated using an HA-tag antibody that recognized RPTPβ from cells expressing both proteins. In order to map the region in RPTPβ that mediates its binding to MAGI-3, we used GST-fusion proteins containing the entire cytoplasmic domain of RPTPβ (βD12), the first phosphatase domain (βD1), the second phosphatase domain (βD2) or a mutant form lacking the C-terminus (βD12dCT) of this phosphatase in pulldown experiments. Cell lysates of HEK-293T cells expressing MAGI-3 were mixed with the various GST-fusion proteins immobilized on beads, and bound proteins were detected by immunoblotting with an antibody to MAGI-3. As shown in Fig. 3B, MAGI-3 was precipitated with βD12 and βD2, but not with βD1 or βD12dCT. Furthermore, MAGI-3 was precipitated from rat brain membrane lysates using a peptide containing the last 12 amino acids of RPTPβ (βCT) immobilized on beads but not by a peptide containing a scrambled sequence (βCTS), nor by a Caspr2 peptide, which contains a type II PDZ-binding sequence (Spiegel et al., 2002) (Fig. 3C). Yeast two-hybrid analysis using the different RPTPβ constructs and the original MAGI-3 fragment obtained from the screen, which contained PDZ3 and PDZ4, yielded similar results (Fig. 3D). Taken together, these results show that MAGI-3 and RPTPβ are found in a protein complex when expressed in the same cells and that this association is mediated by binding of the C-terminal sequence of RPTPβ to the third or fourth PDZ domain of MAGI-3.

Cell-type-specific localization of MAGI-3 at cell junctions, focal adhesions and the nucleus

Proteins containing PDZ domains have been suggested to play a role in the organization of protein complexes at the plasma membrane (Fanning and Anderson, 1999). Accordingly, many of these proteins were found in specialized cell-cell contacts such as adherens and tight junctions (Gonzalez-Mariscal et al., 2000; Nagafuchi, 2001). In order to determine the subcellular localization of MAGI-3, we examined its distribution in epithelial cells and astrocytes. Double labeling of Caco2 colon carcinoma cells with a MAGI-3-specific antibody and antibodies to either ZO-1 or cingulin revealed that MAGI-3 colocalized with these proteins at tight junctions (Fig. 4). In contrast, MAGI-3 only partially overlapped with β-catenin and desmoplakin, two proteins that reside in adherens junctions and desmosoms, respectively. A similar localization of MAGI-3 was detected in Caco2 cells using a different monoclonal antibody (Fig. 4M). Furthermore, MAGI-3 was concentrated at cell-cell contacts in transfected MDCK cells, which lack endogenous MAGI-3 (Fig. 4N,O). In addition, strong punctuate staining of MAGI-3 was found in the nucleus, whereas most of the cell cytoplasm remained unstained or only very weakly labeled. Membrane and nuclear localization of MAGI-3 was also found by cell fractionation (data not shown). Localization of MAGI-3 to tight junctions and the nucleus in Caco2 cells was also confirmed by immunoelectron microscopy (Fig. 5). In addition, gold particles were frequently detected in the cell microvilli, demonstrating that in addition to tight junctions, MAGI-3 is also found at the apical membrane.

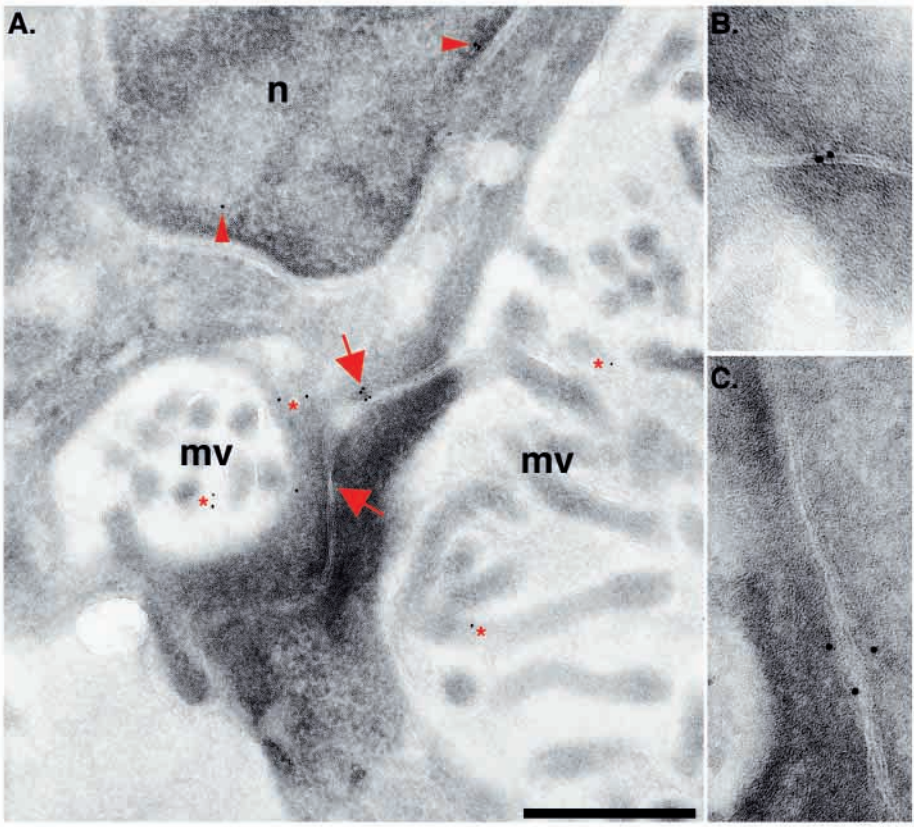

Fig. 5. Ultrastructural localization of MAGI-3. Caco-2 cells were labeled with an antibody to MAGI-3 and a colloidal gold-conjugated secondary goat anti-rabbit antibody. (A) An immunoelectron micrograph showing labeling of the gold particles at tight junctional complex region (red arrows), nucleus (n; red arrowheads) and microvilli (mv: red asterisks) are presented. (B,C) Higher magnification images, showing the localization of MAGI-3 at tight junctions. Bar, A, 200 nm; B,C, 35 nm.

In primary rat astrocytes, a cell type that does not show any tight junction structures in culture, MAGI-3 was found in the nucleus and was localized with β-catenin in cell-cell contacts (Fig. 6A-C). Double labeling of MAGI-3 and anti-phosphotyrosine antibodies showed that MAGI-3 was concentrated along with tyrosine-phosphorylated proteins at sites of cell contacts but was absent in phosphotyrosine-positive focal adhesion sites at the cell's periphery (Fig. 6D-F). At cell-cell contacts, MAGI-3 appeared as a thin discontinuous line at the cell's junction or as a rod-like shape, labeling extended protrusions between neighboring cells. Surprisingly, in older cultures of astrocytes, MAGI-3 was also found in focal adhesions, where it colocalized with vinculin at the edge of actin filaments (Fig. 6G-L). Localization of MAGI-3 to focal adhesion sites was more prominent in dispersed rather than crowded cultures (data not shown). Thus, we concluded that MAGI-3 is found in the cell nucleus and in specific structures along the plasma membrane. Depending on the cell type and culture conditions, it could be found in tight and adherens junctions, cellular protrusions and focal adhesion sites.

Association of MAGI-3 with tyrosine-phosphorylated proteins

The association of MAGI-3 with RPTPβ raises the question of whether it serves as a substrate for this tyrosine phosphatase. To determine whether MAGI-3 could be phosphorylated on tyrosine residues, we treated SF763T cells with sodium orthovanadate, a cell-permeable inhibitor of tyrosine phosphatases that induces a general increase in tyrosine phosphorylation of proteins within the cell (Gordon, 1991). Such treatment caused a marked shift in the apparent molecular weight of MAGI-3 in these cells, as detected by immunoprecipitating MAGI-3 with two different antibodies (Fig. 7A, upper panel). However, this shift was not due to phosphorylation on tyrosine residues, since MAGI-3 was neither detected by anti-phosphotyrosine after immunoprecipitation with an anti-MAGI-3 antibody nor by anti-MAGI-3 immunoblotting after immunoprecipitation with anti-phosphotyrosine antibodies. Furthermore, treatment of MAGI-3 immunocomplexes with alkaline phosphatase did not change its mobility, indicating that the observed shift in MAGI-3 upon vanadate treatment was not due to its phosphorylation either on tyrosine or other residues. Similar results were obtained using potato acid phosphatase (data not shown). Nevertheless, anti-phosphotyrosine immunoblots revealed the presence of a 130 kDa protein in MAGI-3 immunocomplexes from vanadate-treated cells (Fig. 7A, lower panel). A very low level of phosphorylated p130 was occasionally detected in non-treated cells. Importantly, this protein was detected when two distinct MAGI-3 antibodies were used for immunoprecipitation and disappeared after treatment of the immunocomplexes with alkaline phosphatase. Furthermore, similar analysis done with various cell lines demonstrated that MAGI-3 was associated with different tyrosine-phosphorylated proteins (Fig. 7B, lower panel). Although MAGI-3 was associated with a tyrosine-phosphorylated p130 in SF763T and C6 cells, in Caco2 cells it was associated with a 90 kDa protein (Fig. 7B). The identity of these proteins is presently unknown, although it should be noted that it was not recognized by antibodies to several

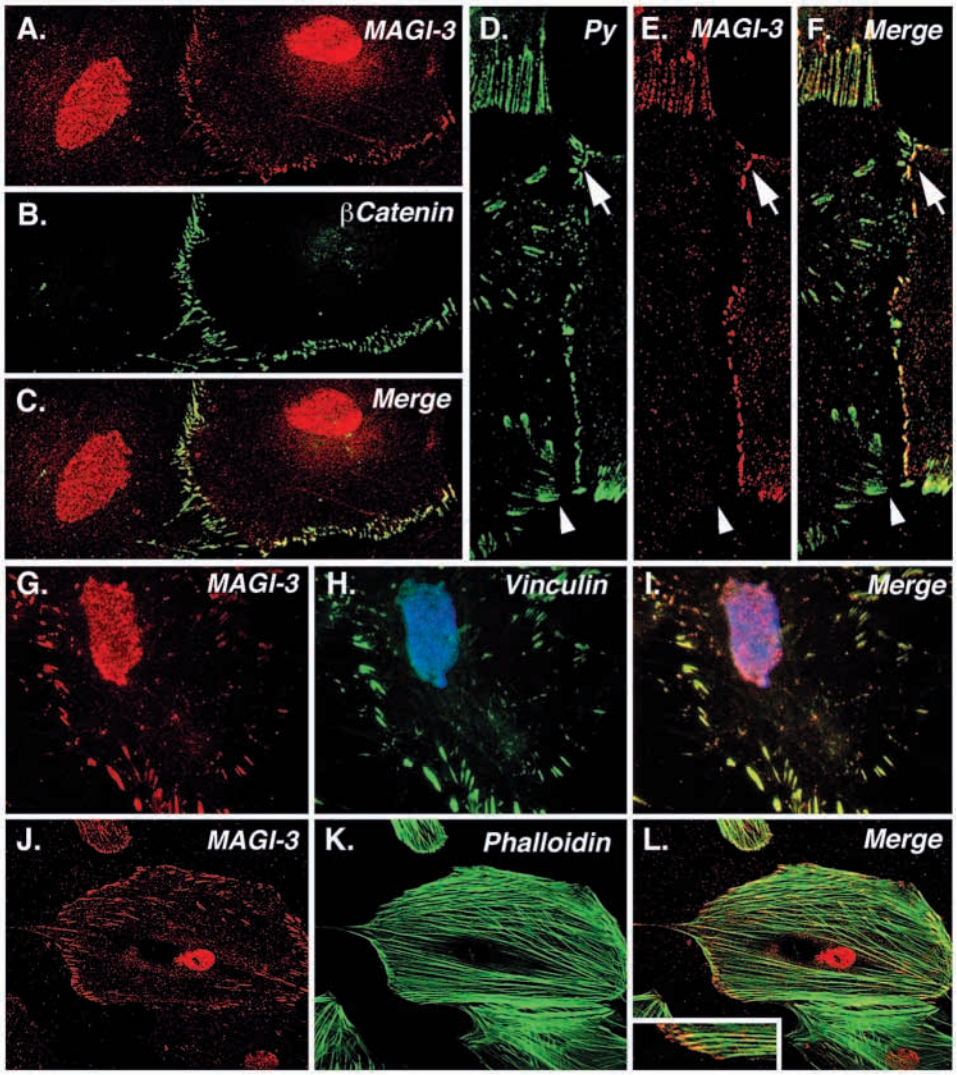

Fig. 6. Immunolocalization of MAGI-3 in primary cultured astrocytes. Primary rat astrocytes (A-F) or older cultures (G-L) were double labeled with antibodies to MAGI-3 (red) and β-catenin (green), phosphotyrosine (PY, green), vinculin (green) or Alexa-488 phalloidin to stain actin filaments as indicated in each panel. Merged images for each corresponding set of staining are shown in the right panels. Colocalization of MAGI-3 and tyrosine phosphorylated proteins at cell-cell contact sites are labeled with an arrow. Arrowheads in D-I mark focal adhesion sites, which contain tyrosine-phosphorylated proteins but not MAGI-3. Localization of MAGI-3 at the end of actin filaments is shown at higher magnification in the inset of the last panel.

candidates such as FAK, p130-Cas, Pyk2 and β-catenin (data not shown).

Dephosphorylation of MAGI-3-associated p130 by RPTPβ

The results so far suggest that MAGI-3 functions as a scaffolding protein linking tyrosine phosphatases such as RPTPβ with their phosphorylated protein substrates. In order to check this possibility, we examined whether phosphorylated p130 could be dephosphorylated by RPTPβ. To this end, we immunoprecipitated MAGI-3 from vanadate-treated SF763T cells and incubated the immunocomplexes with GST-fusion proteins containing the cytoplasmic region of RPTPβ (βD12), a catalytically inactive RPTPβ mutant (βD12DA), a deletion mutant lacking the C-terminal tail of RPTPβ (βD12dCT) or a GST-fusion containing only the first phosphatase domain (βD1) before analyzing them by anti-phosphotyrosine or anti-MAGI-3 antibodies. As depicted in Fig. 8A, p130 was completely dephosphorylated by βD12 and, as expected, not by the catalytically inactive βD12DA. Surprisingly, p130 was not dephosphorylated by βD1 or βD12dCT, although both of these constructs were active phosphatases as determined using nitrophenyl phosphate as a substrate (Fig. 9C). These results demonstrated that p130 could serve as a substrate for RPTPβ. Furthermore, dephosphorylation of p130 required the C-terminal tail of the phosphatase, suggesting that the interaction of this region with MAGI-3 regulates the activity of RPTPβ toward its substrates.

Discussion

In this study we have identified and characterized MAGI-3 (originally termed Slipr) as a protein that interacts with RPTPβ. Rat MAGI-3 is a member of the MAGI family of scaffolding proteins, which includes MAGI-1/BAP1 (Dobrosotskaya et al., 1997; Shiratsuchi et al., 1998), human MAGI-2/AIP/ARIP, rat S-SCAM (Hirao et al., 1998; Shoji et al., 2000; Wood et al., 1998) and human MAGI-3 (Wu et al., 2000b). Similar to other MAGI members, MAGI-3 contains multiple domains involved in protein-protein interactions, including six PDZ domains, a guanylate kinase domain and two WW domains. This domain organization enables members of this family to interact with various cell adhesion molecules (Hirao et al., 1998), receptor kinases (Shoji et al., 2000; Strochlic et al., 2001) and G-protein-coupled receptors (Shiratsuchi et al., 1998; Xu et al.,

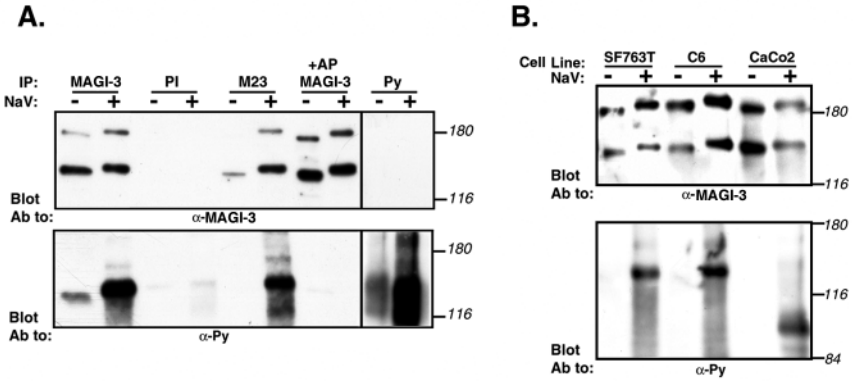

Fig. 7. Association of MAGI-3 with tyrosine-phosphorylated proteins. (A) SF763T cells treated with (+) or without (–) vanadate were immunoprecipitated using antibodies to MAGI-3 (MAGI-3 or M23), preimmune serum (PI) or anti-phosphotyrosine (PY). The immunocomplexes were separated on SDS gels and immunoblotted with anti-MAGI-3 (upper panel) or anti-phosphotyrosine (lower panel) antibodies, as indicated. The immunocomplexes from one set of samples were treated with alkaline phosphatase (MAGI-3 + AP) before immunoblotting. MAGI-3 was neither detected by anti-phosphotyrosine after immunoprecipitation with an anti-MAGI-3 antibody nor by anti-MAGI-3 immunoblotting after immunoprecipitation with anti-phosphotyrosine antibodies. The location of molecular weight markers is shown in kDa. (B) MAGI-3 is associated with different tyrosine-phosphorylated proteins in several cell lines. MAGI-3 was immunoprecipitated from untreated (–) or vanadate-treated (+) SF763T and C6 glioblastoma cells or Caco2 colon carcinoma cells, followed by immunoblotting with anti-phosphotyrosine (lower panel) or anti-MAGI-3 (upper panel) antibodies.

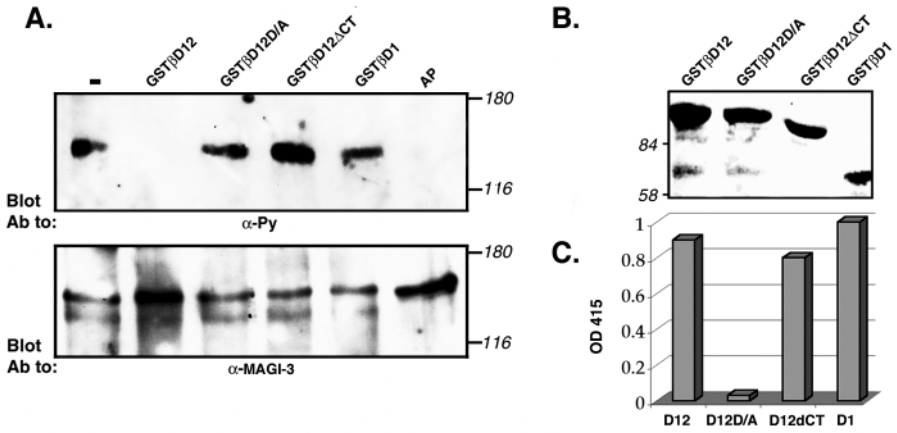

Fig. 8. Dephosphorylation of p130 by RPTPβ. (A) MAGI-3 immunocomplexes from vanadate-treated SF763T cells were incubated with GST or GST-fusion proteins containing the cytoplasmic domain of RPTPβ (βD12), a catalytic inactive mutant (βD12DA), the first phosphatase domain (βD1) or a mutant form lacking the C-terminus (βD12dCT) as indicated. The reaction was terminated by the addition of SDS gel loading buffer, and the samples were immunoblotted with anti-phosphotyrosine (upper panel) or anti-MAGI-3 (lower panel) antibodies. Treatment of the immunocomplexes with alkaline phosphatase (AP) was used as a positive control. (B) Coommassie blue staining of the RPTPβ constructs used in panel A. (C) Phosphatase activity. The indicated RPTPβ GST-fusion proteins were incubated with *p*-nitrophenilphosphate as described in experimental procedures, and the absorbance of the reaction product was measured at 450 nm.

2001). This is the first study that describes an interaction between a MAGI protein and a receptor tyrosine phosphatase. Using two-hybrid analysis, GST-pulldown and peptide pulldown experiments with transfected cells, we found that the association of MAGI-3 with RPTPβ is mediated by binding of the C-terminal tail of the phosphatase to the third or fourth PDZ domain of MAGI-3. The C-terminus of RPTPβ contains a type I PDZ domain binding sequence (i.e. S/TXV) found in proteins that interact with the first, third and fifth PDZ domains of different MAGI family members (Hirao et al., 1998; Patrie et al., 2001; Shoji et al., 2000; Wu et al., 2000b; Xu et al., 2001), raising the possibility that RPTPβ binds more than a single PDZ domain of MAGI-3. In addition, we have found that RPTPβ interacts with the third and fourth PDZ domains of the related S-SCAM/MAGI2 (data not shown). These results suggest that the high sequence similarity shared between different MAGI proteins most probably enables them all to interact with RPTPβ, as well as with its close relative RPTPγ, which contains an identical C-terminal sequence (Barnea et al., 1993).

The dynamic arrangement of cell-cell contacts is important for maintaining cell polarity and allowing communication between neighboring cells by precise deposition of receptors and their downstream effectors at the plasma membrane (Fanning and Anderson, 1999). Proteins containing PDZ domains have been localized to tight, adherens and synaptic junctions, where they interact with the cytoskeletal proteins, thereby providing a direct link between membrane signals and cell morphology (Gonzalez-Mariscal et al., 2000; Nagafuchi, 2001). We have found that in epithelial cells, MAGI-3 is localized with ZO-1 and cingulin to tight junctions, whereas in primary astrocytes it was found with β-catenin in adherens junctions and cellular protrusions. The latter are reminiscent of the dynamic finger-like structures observed when keratinocytes in culture make contacts (Vasioukhin et al., 2000). Our results are in agreement with studies describing the subcellular localization of MAGI-1, which was found at tight junctions in MDCK cells (Dobrosotskaya and James, 2000; Ide et al., 1999), as well as in cadherin-based adherens junctions in normal kidney NRK cells (Nishimura et al., 2000). However, in contrast to other family members, MAGI-3 was also found in focal adhesion sites in aged astrocyte cultures, suggesting that its subcellular localization not only depends on the cell type, but also on the culture condition. In addition to its localization to specific sites at the plasma membrane, MAGI-3 was strongly expressed in the nucleus. MAGI-3 contains two

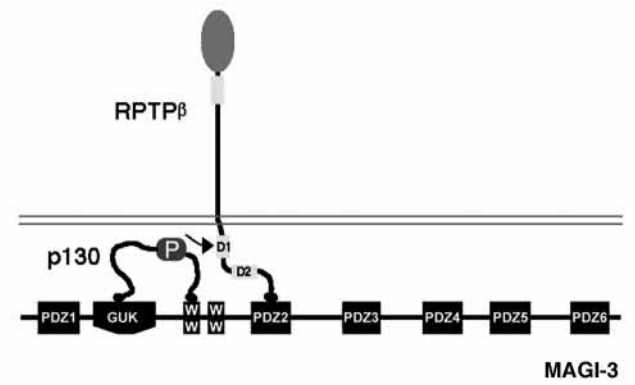

Fig. 9. Schematic model of RPTPβ/Slipr complex. The C-terminal tail of RPTPβ binds to the third PDZ domain of Slipr/MAGI-3. This interaction brings the phosphatase into proximity with its tyrosine phosphorylated p130 substrate, resulting in dephosphorylation of p130. Since the molecular identity of p130 is not known, it was arbitrarily drawn bound to one PDZ and one WW domain. It is likely that the multi-domain organization of MAGI-3 allows it to bind simultaneously to other proteins in addition to RPTPβ and p130, generating a signaling complex at the plasma membrane.

putative nuclear localization signals located at its N-terminus (KKKKH at aa 6) and in its guanylate kinase domain (KRKK at amino acid 368). Importantly, nuclear localization of MAGI-3 was detected in all cell types examined using two distinct antibodies and was also confirmed by biochemical fractionation of the cells (data not shown). In this regard, the nuclear localization of MAGI-3 is similar to several other junctional proteins containing PDZ domains that could also be found in the nucleus (Dobrosotskaya et al., 1997; Gottardi et al., 1996; Hsueh et al., 2000). It was suggested that the binding of these proteins to transmembrane proteins sequester MAGI at the plasma membrane, thereby regulating their nuclear localization. Furthermore, two of these proteins, ZO-1 and CASK, were recently shown to activate gene expression by binding to the Tbr-1 and ZONAB transcription factors, respectively (Balda and Matter, 2000; Hsueh et al., 2000). Although transcriptional activity of MAGI-3 remains to be explored, the subcellular localization of MAGI-3 raises the possibility that it takes part in a signaling pathway that is regulated by cell adhesion and cell-cell contact.

Cell adhesion and cell-cell contact are controlled by tyrosine phosphorylation. We showed that treatment of cells with vanadate induced a marked shift in the apparent molecular mass of MAGI-3. However, the nature of this modification is presently not clear. Despite the presence of a conserved tyrosine residue at position 356 in MAGI-3, which is phosphorylated by Src in MAGI-1 (Nishimura et al., 2000), we found no indication that MAGI-3 is tyrosine phosphorylated. Instead, vanadate treatment revealed that MAGI-3 was associated with few tyrosine-phosphorylated proteins. A 130 kDa tyrosine-phosphorylated protein that was associated with MAGI-3 in most of the cell lines examined served as a substrate for RPTPβ. Interestingly, we found that p130 could serve as a substrate for RPTPβ. Strikingly, dephosphorylation of p130 by RPTPβ required the C-terminal tail of this phosphatase. Given that this region mediates the interaction of RPTPβ with MAGI-3, these results suggest that the latter may function as a scaffolding protein that bridges between the phosphatase and p130 (Fig. 9). Furthermore, the multi-domain organization of MAGI-3 may allow it to establish higher order signaling complexes, as suggested for other multi-PDZ-containing proteins (Harris and Lim, 2001). In the case of RPTPβ such a signaling complex may consist of a mesh of several distinct scaffolding proteins including members of the MAGI and PSD95 families. Accordingly, it was shown that the MAGI-3-related S-SCAM/MAGI-2 interacts with PSD95 and β-catenin, two proteins that are found in a complex with RPTPβ (Dobrosotskaya and James, 2000; Kawachi et al., 1999). Our results suggest that the association of RPTPβ with MAGI-3 may allow the recruitment of p130 and additional substrates of this phosphatase. Interestingly, it was recently demonstrated that both S-SCAM/MAGI-2 and MAGI-3/MAGI-3 are found in a complex with the tumor suppressor PTEN, and they regulate its activity by recruiting it to the plasma membrane where its PtdIns(3,4,5)P_3 phospholipid substrate is localized (Wu et al., 2000a; Wu et al., 2000b). Similarly, PTPμ interacts with RACK (receptor for activated protein C kinase), a scaffolding protein that is thought to recruit proteins to the plasma membrane (Mourton et al., 2001). The above data, along with the results presented here, suggest that the use of scaffolding proteins such as MAGI-3 to bring tyrosine-phosphorylated proteins in close proximity to their phosphatases, may be a general mechanism that regulates the function of these enzymes.

This work was supported by the Israeli Academy of Science, the Yad Abraham Research Center for Cancer Diagnostics and Therapy and a Career Development award from the Israeli Cancer Research Foundation. E. P. is Incumbent of the Madeleine Haas Russell Career Development Chair.

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