MALS is a binding partner of IRSp53 at cell–cell contacts

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Abstract Insulin receptor substrate p53 (IRSp53) is a key player in cytoskeletal dynamics, interacting with the actin modulators WAVE2 and Mena. Here, we identified a PDZ protein, MALS, as an IRSp53-interacting protein using a yeast two-hybrid screen. A pull-down assay showed that IRSp53 and MALS interact through the PDZ domain of MALS and the C-terminal PDZ-binding sequence of IRSp53. Their interaction in MDCK cells was also demonstrated by co-immunoprecipitation. Immunocytochemistry showed the colocalization of IRSp53 and MALS at cell–cell contacts. Cytochalasin D induced the redistribution of both proteins to the cytosol. Thus, MALS is a partner of IRSp53 anchoring the actin-based membrane cytoskeleton at cell–cell contacts.

Key words: PDZ domain; Actin modulator; Membrane cytoskeleton; Epithelial cell

1. Introduction

Insulin receptor substrate p53 (IRSp53) was originally identified as an insulin/insulin-like growth factor stimulation-dependent tyrosine-phosphorylated protein in CHO cells and as one of the postsynaptic density (PSD) components. [1,2]. This protein differs from other insulin receptor substrate (IRS) family members in its conserved domain structure [3]. Four isoforms of IRSp53 (IRSp53L, S, T, and IRS-58) have been identified; each is composed of a common N-terminal portion (amino acids 1–511) containing several protein–protein-interacting domains and a short, unique C-terminal region [4]. Although different tissue distributions and phosphorylation among the L, S, and T isoforms have been reported [4], how their functions differ remains unknown. One recent study demonstrated the involvement of IRSp53 in actin reorganization. When Rac interacts with the N-terminus of IRSp53, the src homology 3 (SH3) domain of IRSp53 binds to WAVE2, resulting in the synergistic promotion of membrane ruffling [5]. Cdc42 also binds to part of the CRIB (Cdc42/Rac-interactive binding) domain of IRSp53, inducing the interaction of the SH3 domain of IRSp53 with Mena (an Ena/VASP family member), followed by the activation of F-actin-enriched filopodial and neurite extensions [6,7].

Here, we used a yeast two-hybrid screen to identify the mammalian Lin-7 homologue MALS/Velis as an IRSp53S-interacting protein, and demonstrated a direct interaction between these proteins by in vitro binding assays. Further, we demonstrated a physiological interaction between these two proteins in the actin-based membrane cytoskeleton at cell–cell contacts between MDCK cells, by co-immunoprecipitation and immunocytochemistry.

2. Materials and methods

2.1. Plasmid construction

Rat IRSp53S cDNA (NM_057196) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and ligated into the pcDNA3.1(+)-FLAG vector (Invitrogen). The cDNA encoding amino acid (aa) residues 1–460 of IRSp53S was amplified by PCR and ligated into pGEX6P2 (Amersham Pharmacia Biotech). The cDNA encoding the full-length (aa 1–207) rat MALS2 obtained by a yeast two-hybrid screen was subcloned into pGEX6P3. The truncated mutants of IRSp53S and MALS2 (Fig. 1) were amplified by PCR and ligated into pGEX6P2 or pcDNA3.1(+)-FLAG vectors, respectively. All constructs were sequenced using a 310 Genetic Analyzer (ABI Prism).

2.2. Cell culture and transfection

COS7 and MDCK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. COS7 cells were transfected using TransIT-LT1 (Mirus). For biochemical analyses, COS7 cells were harvested 36 h after transfection.

2.3. Antibody production

Polyclonal antibodies against IRSp53 and MALS were produced as follows: glutathione *S*-transferase (GST)-fused IRSp53 (aa 1–460) and MALS2 (aa 1–207) expressed in *Escherichia coli* BL21 were purified using a glutathione-conjugated Sepharose 4B column (Amersham Pharmacia Biotech), and were used for immunization into New Zealand White rabbits. Antisera were preabsorbed with GST protein, followed by purification using a GST-fused IRSp53 or MALS2-coupled Sepharose 4B gel matrix, respectively.

2.4. Yeast two-hybrid screens

Rat IRSp53S cDNA (encoding aa 19–522) was fused to the LexA DNA-binding domain using the pBTM116 vector. IRSp53S bait plasmid was used to screen a rat brain cDNA library cloned into the pACT2 vector (Clontech). To select the putative interacting proteins, yeast strain L40 transformed by the bait plasmid and the cDNA library was grown on a yeast selection plate containing 5 mM 3-aminotriazole and lacking tryptophan, leucine, and histidine. The acquired colonies were tested for β-galactosidase activity. The library plasmids obtained by this screening were recovered through *E. coli* transformation. The insert DNA in the positive clones was sequenced.

2.5. Immunocytochemistry

MDCK cells cultured on coverslips were fixed in 3.7% formaldehyde for 20 min, then permeabilized with 0.25% Triton X-100, and incubated in blocking solution containing 5% normal goat serum, 1% bovine serum albumin, and 0.05% Triton X-100 in phosphate-buffered saline minus (PBS) for 30 min. The following primary antibodies were

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Abbreviations: IRSp53S, insulin receptor substrate p53 short form; PDZ domain, PSD-95/Disc large/ZO-1 homology domain; MALS, mammalian Lin-7 homologue

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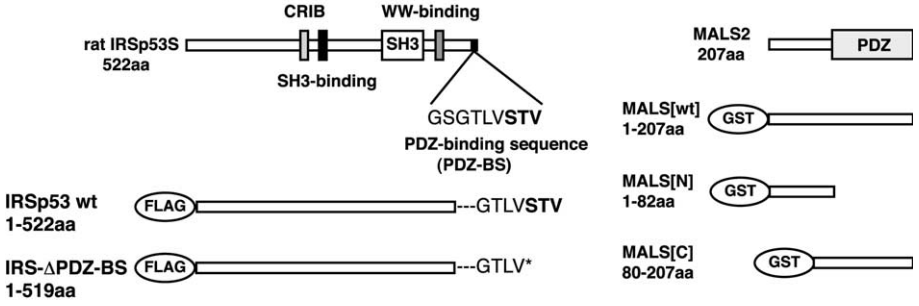


Fig. 1. Schematic diagrams of the IRSp53S and MALS domain structures. IRSp53 contains several protein–protein-interaction domains: from the N-terminus, a partial CRIB domain, SH3-binding domain, SH3 domain, WW-binding domain, and putative PDZ-binding sequence at the C-terminal end. MALS2 has a unique N-terminal sequence followed by a PDZ domain that is highly homologous among the MALS family members. The truncated mutants used in this experiment are also shown.

incubated overnight in blocking solution: anti-IRSp53 or -MALS polyclonal antibody (1:200) and anti-β-catenin (clone 14, Transduction Laboratories) monoclonal antibody (1:500). F-actin was labeled with Alexa Fluor™ 568-phalloidin. Subsequently, the cells were labeled with Alexa Fluor™ 488-conjugated anti-rabbit and 546-conjugated anti-mouse secondary antibodies (2 μg/ml, Molecular Probes) in the blocking solution for 40 min. After being washed with PBS, the coverslips were mounted onto glass slides using a Prolong Antifade Kit (Molecular Probes). Where indicated, the cells were extracted with Triton X-100 buffer (50 mM NaCl, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, and 300 mM sucrose) on ice for 10 min before fixation [8]. The confocal images were obtained using a Zeiss 410 confocal laser scanning microscope.

2.6. Biochemical analysis

All procedures were performed at 4°C. For the pull-down assay, transfected COS7 cells were lysed in buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a cocktail of protease inhibitors). After centrifugation at 15 000×*g* for 20 min, the supernatant was incubated with control (GST) or GST-fused MALS2 with glutathione-Sepharose 4B beads for 3 h. After being washed with lysis buffer, the proteins bound to Sepharose beads were solubilized with sodium dodecyl sulfate (SDS) sample buffer. For co-immunoprecipitation, MDCK cells were homogenized in solubilization buffer (25 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, and a cocktail of protease inhibitors) for 30 min. After centrifugation, the supernatant was incubated with anti-MALS antibody (5 μg)-conjugated Sepharose 4B (Amersham Pharmacia Biotech) for 4 h. After being washed with solubilization buffer, the proteins bound to Sepharose beads were solubilized with SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Anti-IRSp53 (1:5000), -MALS (1:5000), and -FLAG (1:2000, IBI-Kodak) antibodies were used as the primary antibodies and visualized using peroxidase-conjugated secondary antibodies followed by ECL (Amersham Pharmacia). For the preabsorption of antibodies, the diluted anti-IRSp53 and -MALS antibodies were preincubated with excess amounts of GST-IRSp53 and GST-MALS, respectively.

2.7. cDNA cloning

A cDNA library was constructed from MDCK cells using a ZAP Express™ cDNA synthesis kit (Stratagene). Approximately 1×10^5 plaques of a cDNA library were screened using a ^{32}P-labeled probe encoding the open reading frame of rat IRSp53 cDNA.

3. Results

We isolated IRSp53S using a PSD protein-specific monoclonal antibody (149H) that recognizes the 58/53-kDa protein (unpublished data). To isolate an IRSp53-interacting protein, we performed a yeast two-hybrid screen using rat IRSp53S (aa 19–522) as bait. Among ∼1.1×10^6 clones of a rat brain cDNA library, 17 candidates were isolated (Table 1). Many of them had at least one PSD-95/Disc large/ZO-1 homology (PDZ) domain [9]. We noted the presence of a PDZ-binding-like sequence (S-T-V) at the C-terminal end of IRSp53S. This sequence is conserved among rat (NM_057196), mouse (AAK68153), dog (cloned in this study, AB112349), and human (BAA36586) homologs.

To examine the interaction between IRSp53S and MALS2/Veli-2, which was represented by a large number of the isolated clones, we performed a pull-down assay with GST or GST-MALS2 in extracts from COS7 cells transfected with FLAG-tagged IRSp53S wild type or IRSp53S lacking the C-terminal PDZ-binding sequence (FLAG-IRSp53SΔPDZ-BS) (Fig. 1). FLAG-IRSp53S bound to GST-MALS2, but FLAG-IRSp53SΔPDZ-BS did not (lanes 1–3 in Fig. 2). Further, the PDZ domain of MALS2 (GST-MALS2[C]) bound to FLAG-IRSp53S, but the N-terminal region of MALS2 (GST-MALS[N]) did not (lanes 4 and 5 in Fig. 2). These results indicate that IRSp53S directly interacts with the PDZ domain of MALS via its C-terminal PDZ-binding sequence.

It has been reported that MALS1 and 2 are specifically

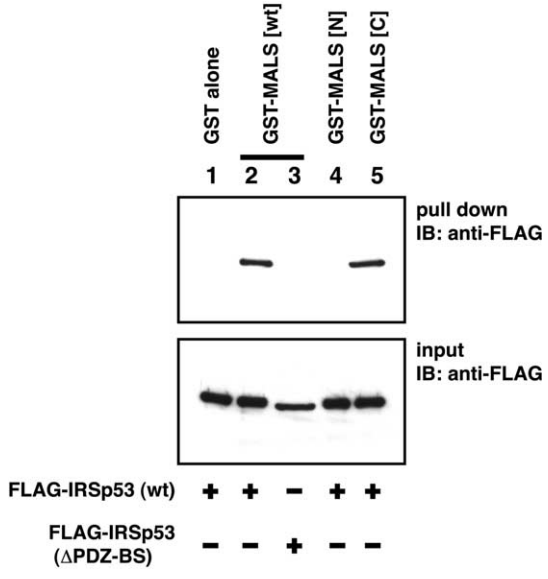


Fig. 2. In vitro interaction of IRSp53S with MALS2. COS7 cells were transfected with expression vectors encoding FLAG-IRSp53S wild type (lanes 1,2,4,5) or FLAG-IRSp53SΔPDZ-BS (lane 3). The cell extracts were pulled down with GST alone (lane 1), GST-MALS2[wt] (lanes 2,3), or truncated mutants GST-MALS2[N] (lane 4), or GST-MALS2[C] (lane 5). The proteins that bound to the above ligands were analyzed by Western blotting.

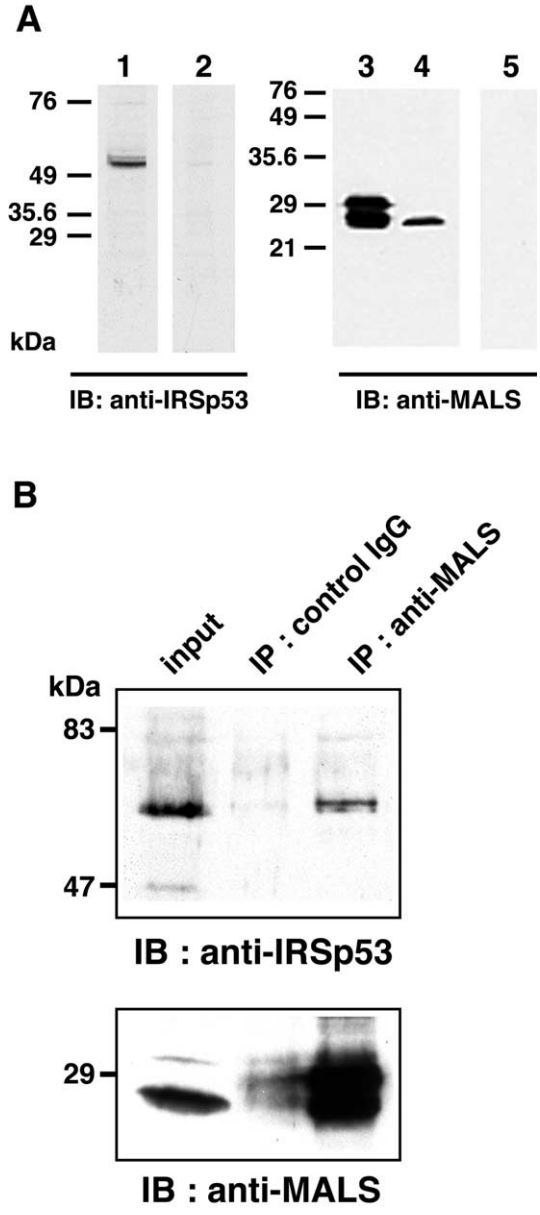


Fig. 3. IRSp53 interacts with MALS in MDCK cells. A: MDCK cell lysates (lanes 1,2,4,5: 20 μg protein) or whole rat brain lysates (lane 3: 20 μg protein) were probed with anti-IRSp53 (lane 1) and -MALS (lanes 3,4) antibodies, or the same antibodies preabsorbed with GST-IRSp53 (lane 2) or GST-MALS2 (lane 5). B: In vivo immunoprecipitation from MDCK cell extracts. The extracts were immunoprecipitated with anti-MALS- or preimmune control IgG-conjugated Sepharose 4B (IP). The immunoprecipitates were blotted with the indicated antibodies (IB).

expressed in the brain, whereas MALS3 is widely distributed in the brain, kidney, liver, and MDCK cells [10,11], and that MALS proteins localize to the cell–cell contacts of MDCK cells [8]. Western blotting revealed that the anti-MALS antibody recognized the two bands with apparent molecular masses of 23 kDa (MALS2/3) and 30 kDa (MALS1) in the rat brain lysates, indicating that this antibody cross-reacts with all MALS isoforms (lane 3 in Fig. 3A). The anti-IRSp53 antibody recognizes all IRSp53 isoforms. Using our anti-IRSp53 and -MALS antibodies, we detected IRSp53 and MALS (MALS2/3) proteins with the expected mobilities in MDCK cell lysates (lanes 1 and 4 in Fig. 3A). Further, these bands were not observed when the antibodies were preabsorbed with their respective antigens (lanes 2 and 5 in Fig. 3A). Taking together our present and other data [11], MALS3 is considered to be the major isoform in MDCK cells. To confirm the presence of the IRSp53S homologue in MDCK cells, a cDNA library constructed from MDCK cells was screened using the full-length rat IRSp53S cDNA as a probe. Among five positive clones isolated, four clones encoded IRSp53S containing the PDZ-binding sequence, and one encoded an IRSp53T homologue lacking the PDZ-binding sequence. To verify the interaction between IRSp53 and MALS in vivo, we performed co-immunoprecipitation assays. Immunoprecipitation of extracts from confluent MDCK cells with the anti-MALS antibody, but not with non-immune IgG, resulted in the co-precipitation of IRSp53 (Fig. 3B).

We next examined the immunolocalization of IRSp53 and MALS in MDCK cells. MALS was predominantly localized to cell–cell contacts, where they were co-localized with β-catenin (Fig. 4B). Although IRSp53 was more diffuse than MALS in the cytoplasm, it was co-localized with β-catenin at cell–cell contacts (Fig. 4A), where it also co-localized with the actin-based membrane cytoskeleton, which stained with fluorescence-labeled phalloidin at cell–cell contacts (Fig. 4C) [12]. To confirm whether IRSp53 was linked to the cytoskeletal fraction at cell–cell contacts, we performed in situ extraction with Triton X-100. When MDCK cells were extracted with 0.5% Triton X-100 before fixation, the cytosolic IRSp53 staining was lost without affecting its localization at cell–cell contacts (Fig. 5B). Treatment with the actin-depolymerizing reagent cytochalasin D induced the redistribution of IRSp53, MALS, and F-actin from the cell–cell contacts to the cytosolic puncta (Fig. 5B, cytochalasin D). These results suggested that IRSp53 is linked to the actin-based membrane cytoskeleton.

4. Discussion

In this study, we demonstrated by in vitro binding assays that IRSp53S directly interacts with the PDZ domain of

Table 1
The candidates for IRSp53-interacting proteins

Protein	Number of positive clones	Properties[a]	Reference
Lin7-A (MALS2)	7	PDZ, TMb, Tr	[13]
SAP102	4	PDZ, SH3, RC, TMb	[22]
Eps8	2	SH3	[23]
NIL-16	1	PDZ, TM	[24]
MAGI-1	1	PDZ	[25]
S-SCAMβ	1	PDZ, TMb	[26]
SAP97	1	PDZ, SH3, RC, TMb	[27]

[a]TM, transmembrane domain; TMb, transmembrane-binding protein; RC, receptor clustering; Tr, involved in trafficking.

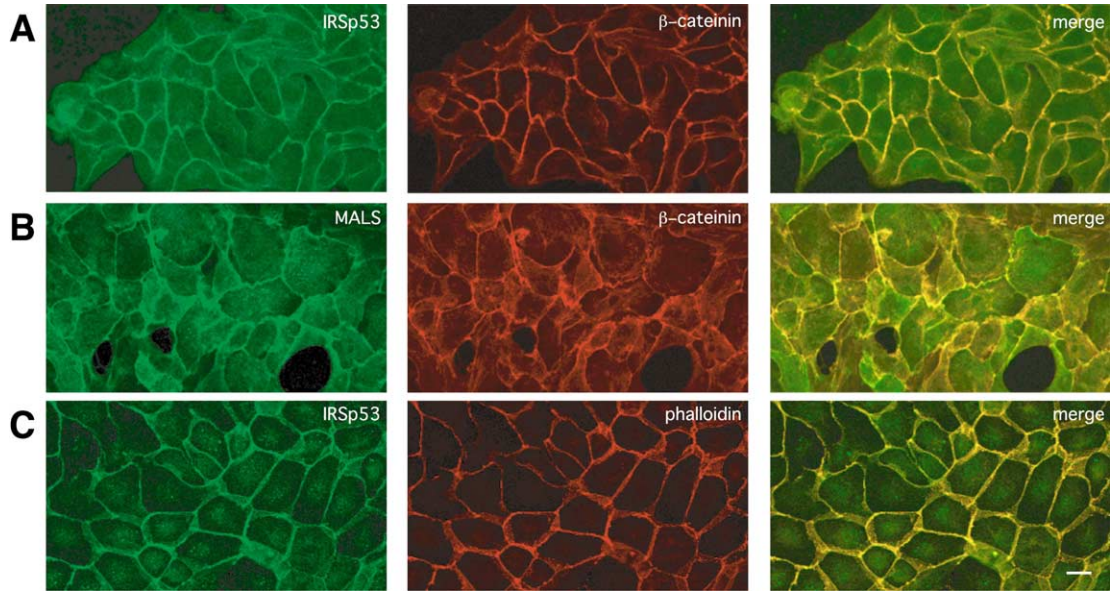
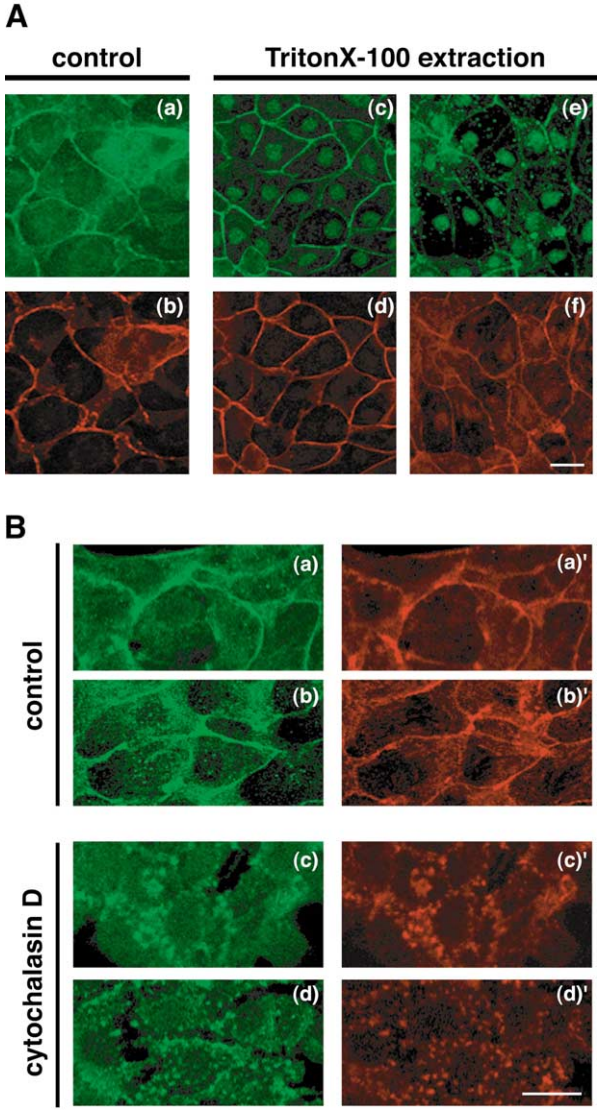


Fig. 4. The localization of IRSp53 and MALS in MDCK cells. MDCK cells were stained with anti-IRSp53 (A,C, green) or anti-MALS (B, green) antibodies. The localization of IRSp53 and MALS was compared with the cell junction marker, β-catenin (A,B, red) or F-actin stained with fluorescence-labeled phalloidin (C, red). Bar, 15 μm.



MALS via its C-terminal PDZ-binding sequence. Jo et al. [10] reported that the PDZ domain of MALS selectively associates with peptides terminating in E-T/S-R/X-V/I/L, by affinity panning using a random peptide library. Indeed, several MALS-interacting proteins, such as the NR2 subunit of *N*-methyl-D-aspartate (NMDA) receptors and LET-23, a receptor tyrosine kinase in *Caenorhabditis elegans*, contain this sequence. Three of the C-terminal residues of IRSp53S fit this sequence, but the fourth amino acid is not glutamate but valine (Fig. 1). Thus, the interaction between IRSp53 and MALS is an exception to this rule, along with the MALS/β-catenin interaction. The C-terminal end of β-catenin is D-T-D-L [8]. We also demonstrated the interaction between IRSp53 and MALS in MDCK cells by co-immunoprecipitation (Fig. 3). MALS1 and 2 are specifically expressed in the brain, whereas MALS3 is expressed in the brain and other, peripheral tissues [10]. The amino acid sequences among the MALS family members are highly conserved (85.7% homology between MALS2 and 3 of total residues, and 95.4% between the PDZ domains of MALS2 and 3), suggesting an interaction between IRSp53S and MALS isoforms is possible.

Confocal imaging analysis showed that IRSp53 and MALS are co-localized at cell–cell contacts of MDCK cells (Fig. 4). In polarized MDCK cells, the IRSp53 at cell–cell contacts was resistant to non-ionic detergent, and treatment with cytochalasin D induced the translocation of IRSp53 from the cell–cell contacts to the cytoplasmic puncta, indicating that IRSp53 is

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Fig. 5. IRSp53 links to the actin-based membrane cytoskeleton. A: MDCK cells were fixed without treatment (a,b) or pretreated with extraction buffer before fixation (c–f). The fixed cells were co-immunostained with anti-IRSp53 (a,c,e) and anti-β-catenin (b,d) or phalloidin (f). B: MDCK cells were incubated with (c,c′,d,d′) or without (a,a′,b,b′) 2 μM cytochalasin D for 2 h. After fixation, the cells were immunolabeled with anti-IRSp53 (a,c; green) or anti-MALS (b,d) antibodies. F-actin was visualized with fluorescence-labeled phalloidin (a′–d′; red). Bar, 15 μm.

a component of the actin-based membrane cytoskeleton (Fig. 5). Strong cell–cell contact requires a linkage between cadherin and the actin cytoskeleton via catenins [13,14]. In addition, members of the Rho family of small G proteins (Rho, Rac1, and Cdc42) and VASP/Mena have been demonstrated to regulate adherens junction formation in MDCK cells and primary epidermal keratinocytes [15–18]. Thus, IRSp53 may work together with its interacting partners in the formation or maintenance of the adhesion structures of epithelial cells. It has been demonstrated that MALS/Lin-7 interacts with several proteins, including the NR2B subunit of the NMDA receptor, β-catenin, BGT-1, LET-23, Pals, and VAM-1, and carries them to appropriate sites such as cell–cell contacts of epithelial cells or synaptic sites of neurons [8,10,19–21]. Thus, MALS may play a role in translocating IRSp53S to or anchoring it at the cell–cell contacts of epithelial cells.

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