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WAVE2 serves a functional partner of IRSp53 by regulating its interaction with Rac☆

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Abstract

We previously reported that IRSp53 binds both Rac and WAVE2, inducing formation of Rac/IRSp53/WAVE2 complex that is important for membrane ruffling. However, recent reports noted a specific interaction between IRSp53 and Cdc42 but not Rac, which led us to re-examine the binding of IRSp53 to Rac. Immunoprecipitation analysis and pull-down assay reveal that full-length IRSp53 binds Rac much less efficiently than the N-terminal fragment, which may be caused by intramolecular interaction. Interestingly, the intramolecular interaction is interrupted by the binding of WAVE2 and full-length IRSp53 associates with Rac in the presence of WAVE2. We also report that IRSp53 induces spreading and neurite formation of N1E-115 cells, which presumably reflect functional cooperation with Rac. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: IRSp53; Rac; WAVE2; Arp2/3 complex; Cdc42; N1E-115 cells

There are five members of the Wiskott–Aldrich Syndrome Protein (WASP) family: WASP, N-WASP, and WAVE/Scar 1, 2, and 3 [1–5]. All possess a verprolin-, cofilin-homology, and the acidic-rich (VCA) domain at their C-termini and activate the Arp2/3 complex, which induces the rapid actin polymerization needed for the formation of protrusive membrane at the leading edges of motile cells [6]. Among the five members, WASP and N-WASP have been studied extensively at the molecular level. WASP and N-WASP form folded structures and are inactive during the resting state because of an intramolecular interaction that is relieved by direct binding of Cdc42, a Rho-family member, and its co-factor phosphatidylinositol 4,5-bisphosphate [7–12]. This unfolding exposes the C-terminal VCA domain and induces activation of the Arp2/3 complex. Recent reports from several laboratories, including ours, have indicated a different mechanism of N-WASP activation by SH3 domain-containing proteins such as Grb2/Ash, WASP-interacting SH3 protein (WISH), and Nck [13–15]. In these cases, the SH3 domain is thought to bind directly to the proline-rich region of N-WASP and induce a conformational change in N-WASP. Collectively, WASP and N-WASP appear to be integrating platforms for various intracellular signals to induce the re-organization of the actin cytoskeleton.

In contrast, WAVE/Scars have been suggested to play a role in the formation of membrane ruffles downstream of Rac, another Rho-family member [3,4]. However, a direct interaction between Rac and WAVEs has not been reported and how the Rac signal is transmitted to WAVEs remained unclear. We searched for proteins that bind to WAVEs and found that IRSp53, which was originally identified as a substrate for the insulin receptor [16], associates directly and specifically with the proline-rich region of WAVE2 [17]. In addition, we also reported that the N-terminal region (Rac-binding region, RCB) of IRSp53 binds directly to activated Rac

☆ Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GFP, green fluorescent protein; GST, glutathione-S-transferase; RCB, Rac-binding; SDS, sodium dodecyl sulfate; VCA, verprolin-, cofilin-homology, and the acidic-rich; WASP, Wiskott–Aldrich syndrome protein.

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[17]. Therefore, IRSp53 appears to be a promising adapter protein between Rac and WAVE2. However, recent reports indicated that IRSp53 binds specifically to Cdc42 but not to Rac [18,19], which prompted us to re-examine the mode of interaction between Rac and IRSp53.

Here we report the results of detailed analyses on the interaction between Rac and IRSp53. We found that full-length IRSp53 has only a very weak ability to bind to Rac. However, when IRSp53 forms a protein complex with WAVE2, it shows a significantly greater ability to bind Rac, probably because of conformational change induced by WAVE2-binding. We also found that IRSp53 induces spreading and neurite formation of N1E-115 cells, which presumably reflect cooperation with Rac.

Materials and methods

Ectopic expression in COS7 cells and N1E-115 cells. COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfection was done with Lipofectoamine (Invitrogen) according to manufacturer's instructions. After 30 h, cells were harvested in lysis buffer (40 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, and protease inhibitors such as aprotinin and leupeptin). N1E-115 cells were also cultured in DMEM/FCS. Transfection was done with Lipofectoamine2000 (Invitrogen) in the presence of serum. After 30 h, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. Cells on coverslips were then subjected to immunofluorescence microscopy as described previously [2].

Antibodies, recombinant proteins, and expression constructs. Anti-Cdc42 (rabbit polyclonal) antibody was purchased from Calbiochem. Anti-Rac (mouse monoclonal) antibody was a gift of Dr. Azuma (Keio University, Japan). Anti-His (mouse monoclonal), anti-Myc (rabbit polyclonal), and anti-FLAG (mouse monoclonal) antibodies were purchased from Santa Cruz and Sigma, respectively. Secondary antibodies linked to alkaline-phosphatase and fluorescein isothiocyanate or rhodamine were from Promega and Capel, respectively.

Glutathione-S-transferase (GST)-fusion proteins of various truncated fragments of IRSp53 were expressed in *Escherichia coli* with pGEX plasmids (Pharmacia Biotech). Protein expression and purification were done as described before [20]. GST-fusion proteins of full-length IRSp53 and non-tagged WAVE2 were expressed in Sf9 cells with recombinant baculoviruses as described previously [17]. Cdc42 and Rac proteins were expressed in *E. coli* as GST-fusion proteins and the GST part was cleaved off by thrombin digestion. GTPγS-loading was done as described previously [7]. The His-tagged fragments of IRSp53 were expressed in *E. coli* with pQE plasmids (Qiagen) and purified with Ni^{2+}–NTA–agarose beads. The expression constructs in mammalian cells were made in pEF-BOS plasmid vector.

Pull-down assay. GST-fusion proteins (20–40 μg) were immobilized on 20 μl glutathione–Sepharose 4B beads (Pharmacia Biotech). Beads were mixed with protein samples, such as GTPγS-loaded Cdc42, Rac, and His-RCB (1 μM), and then incubated with rotation for 2 h at 4 °C. The beads were washed five times in lysis buffer and then suspended in 20 μl sodium dodecyl sulfate (SDS)-sample buffer. Samples (5 μl) were loaded in each lane of an SDS gel and separated electrophoretically. The separated proteins were stained directly with Coomassie Brilliant Blue or transferred to polyvinylidene difluoride membranes for Western blotting analysis.

Results and discussion

Co-immunoprecipitation of the N-terminal fragment of IRSp53 with Rac

To examine the binding between IRSp53 and Rac, we ectopically expressed IRSp53 (Myc-tagged full-length (WT) or RCB) (shown schematically in Fig 1A) with or without activated Rac mutant (FLAG-tagged

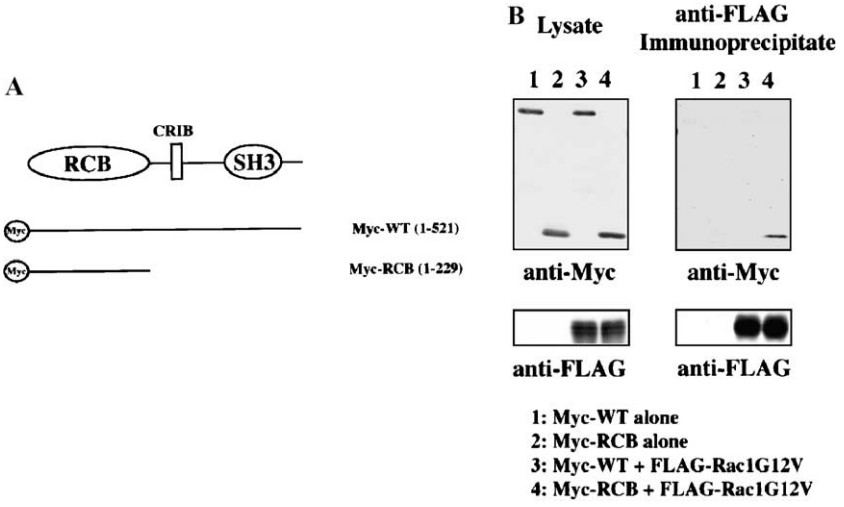

Fig. 1. Co-immunoprecipitation of the N-terminal fragment (RCB) of IRSp53 with Rac. (A) Schematics of full-length (WT) and RCB constructs of IRSp53. The RCB region, the partial CRIB motif, and the SH3 domain are indicated. (B) Co-immunoprecipitation of RCB fragment with Rac. COS7 cells were transfected with Myc-WT or Myc-RCB with or without FLAG-Rac1G12V. Cells were then harvested, and the resulting lysates were subjected to immunoprecipitation with anti-FLAG antibody. The precipitates were examined by Western blotting analysis with anti-Myc and anti-FLAG antibodies.

Rac1G12V) in COS7 cells, and the cell lysates were immunoprecipitated with anti-FLAG antibody. Analysis of the immunoprecipitates by Western blotting with anti-Myc antibody revealed that full-length IRSp53 (WT) did not co-precipitate with Rac under these experimental conditions, whereas clear co-precipitation of the RCB fragment was observed (Fig. 1B). This result indicates that at least the RCB region associates with activated Rac in cells and suggests the possibility that Rac-binding ability may be masked in full-length IRSp53. However, cell lysates contain many other proteins in addition to IRSp53 and Rac, and it is impossible to determine definitively this possibility by immunoprecipitation analysis.

Increased binding between Rac and IRSp53 in the presence of WAVE2

To examine the scenario described above, we next performed an in vitro pull-down assay with purified IRSp53 and Rac proteins. For this purpose, we used various truncated fragments of IRSp53 (Fig. 2A). As shown in Fig. 2B, a strong association with GTPγS-loaded Rac was observed when the GST-CRIB fragment derived from PAK, a serine/threonine kinase that binds strongly to both Cdc42 and Rac, was used as a positive control. In contrast, GST-WT (full-length) of IRSp53 showed only a very weak positive signal. Deletion from the C-terminus of IRSp53 enhanced binding to Rac, and GST-RCB showed a significantly strong signal.

We previously showed that a significant amount of endogenous IRSp53 in NIH3T3 cell lysates is present as a complex with endogenous WAVEs (mostly WAVE2) [17]. Therefore, we tested whether addition of WAVE2 to IRSp53 affected binding to Rac. GST-WT was first immobilized on beads and pre-incubated with lysates derived from Sf9 cells infected with WAVE2-expressing baculoviruses. The beads were washed and then mixed with the GTPγS-loaded Rac proteins. GST-WT pre-incubated with WAVE2 showed comparable Rac-binding to GST-RCB (Fig. 2C), clearly indicating that full-length IRSp53 can associate with Rac when bound to WAVE2.

Possible masking of the RCB region by intramolecular interactions and release by WAVE2

We then investigated the mechanism of how Rac-binding ability is masked in full-length IRSp53. Krugmann et al. [19] recently reported that the N-terminal fragment (amino acids 1–178) of IRSp53 associates intramolecularly with the central region. We thought that this intramolecular interaction might be the basis for the masking of Rac-binding ability in full-length

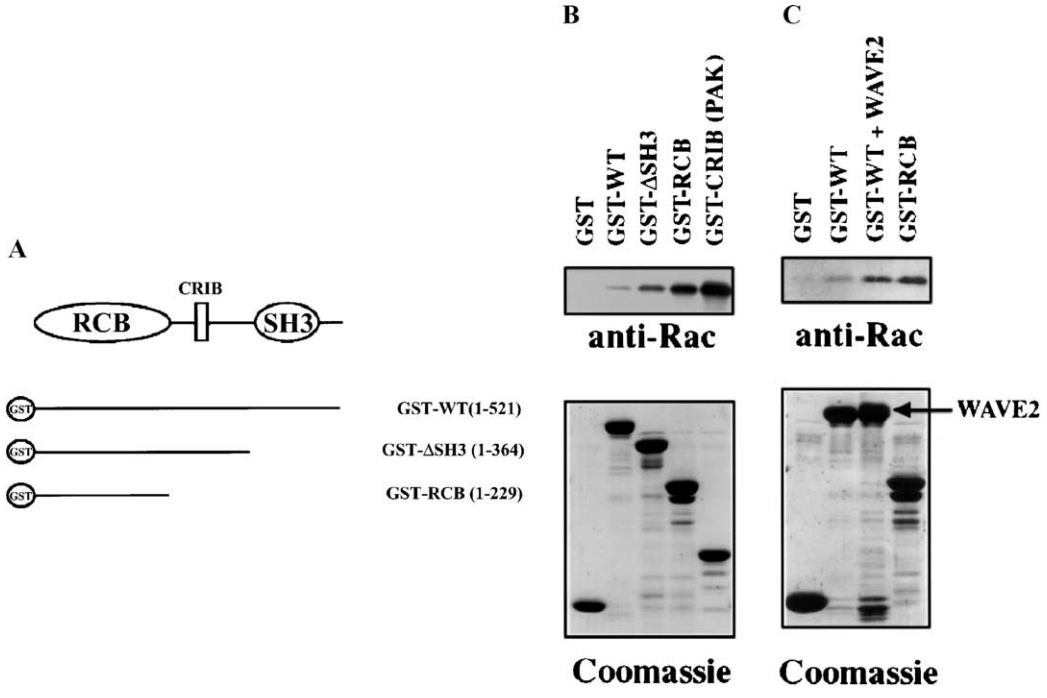

Fig. 2. Increase in binding of Rac to IRSp53 in the presence of WAVE2. (A) Schematics of WT, ΔSH3, and RCB GST-fusion proteins of IRSp53. (B) Binding between various deletion mutants of IRSp53 and Rac. GST-fusion proteins of IRSp53 and the CRIB motif from PAK (used as a positive control) were immobilized on beads and then incubated with 1 μM GTPγS-loaded Rac. After washing, the beads were suspended in SDS-sample buffer and subjected to Western blotting analysis with anti-Rac antibody. (C) Increased binding between Rac and IRSp53 in the presence of WAVE2. GST-WT of IRSp53 immobilized on beads was pre-incubated with lysates from Sf9 cells infected with WAVE2-expressing baculoviruses. After washing, the beads were mixed with GTPγS-loaded Rac.

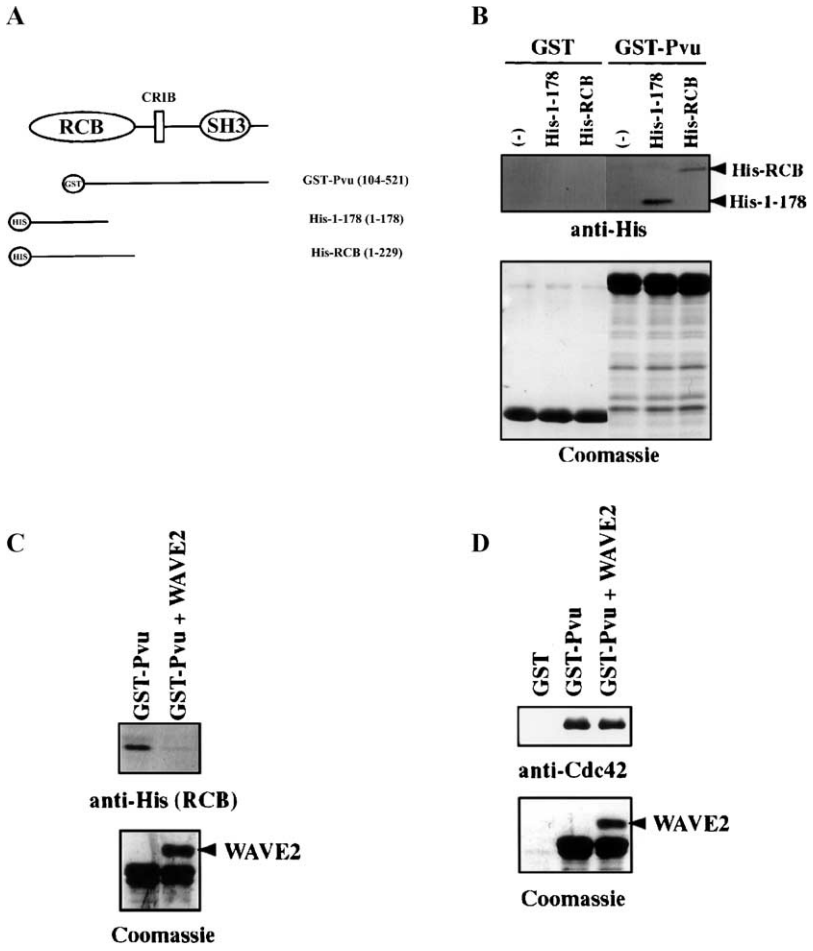

Fig. 3. Possible masking of the Rac-binding region by an intramolecular interaction and its interruption by WAVE2. (A) Schematics of GST-Pvu and the His-tagged proteins of the RCB region and the N-terminal region (1–178) of IRSp53. (B) Binding of His-RCB and His-1–178 to GST-Pvu. GST-Pvu was immobilized on beads and then incubated with 1 μM His-RCB or His-1–178. After washing, the beads were suspended in SDS-sample buffer and subjected to Western blotting analysis with anti-His antibody. (C) Inhibition of binding between His-RCB and GST-Pvu by WAVE2. GST-Pvu proteins immobilized on beads were pre-incubated with WAVE2-expressing Sf9 cell lysates. After washing, the beads were mixed with His-RCB. (D) Binding of Cdc42 to GST-Pvu in a WAVE2-independent manner. GST-Pvu proteins (pre-incubated with or without WAVE2) immobilized on beads were incubated with 1 μM GTPγS-loaded Cdc42. The amount of Cdc42 bound to the beads was examined by Western blotting analysis.

IRSp53. Indeed, we found that the His-tagged portions of the RCB fragment also bound to GST-Pvu lacking the N-terminal 103 residues (Figs. 3A and B).

We then examined whether the binding between GST-Pvu and His-RCB, which may reflect intramolecular masking, is affected by the binding of WAVE2. Prior to mixing with His-RCB proteins, GST-Pvu immobilized on beads was mixed with lysates from WAVE2-expressing Sf9 cells. Under this condition, we observed a significant decrease in the binding to His-RCB (Fig. 3C). This result suggests that WAVE2-binding influences the conformational change in IRSp53, which is consistent with the mechanism of WAVE2-induced binding to Rac described above.

We also examined whether binding of Cdc42 to IRSp53 is affected by WAVE2. The previous reports indicated that Cdc42 binds directly to the partial CRIB motif located in the central region of IRSp53 [18,19]. Indeed, we observed a positive signal of Cdc42-binding to GST-Pvu that contains the motif (Fig. 3D). We next performed a pull-down assay of Cdc42 by GST-Pvu complexed with WAVE2 and found that WAVE2 does not affect the binding of Cdc42 to GST-Pvu (Fig. 3D). These results suggest that Cdc42 can employ both free IRSp53 and IRSp53/WAVE2 complexes as effectors, but Rac can do so only the latter complex.

IRSp53 induces spreading and neurite formation of N1E-115 cells in an SH3 domain-dependent manner

We next investigated the physiological function of IRSp53. It was reported that ectopic expression of IRSp53 induces neurite formation in N1E-115 neuroblastoma cells [18], and thus, we performed ectopic expression analyses using N1E-115 cells. Both untransfected cells (shown by arrows in Fig. 4A, a,b) and the control green fluorescent protein (GFP)-expressing cells (shown by arrowheads) showed a rounded

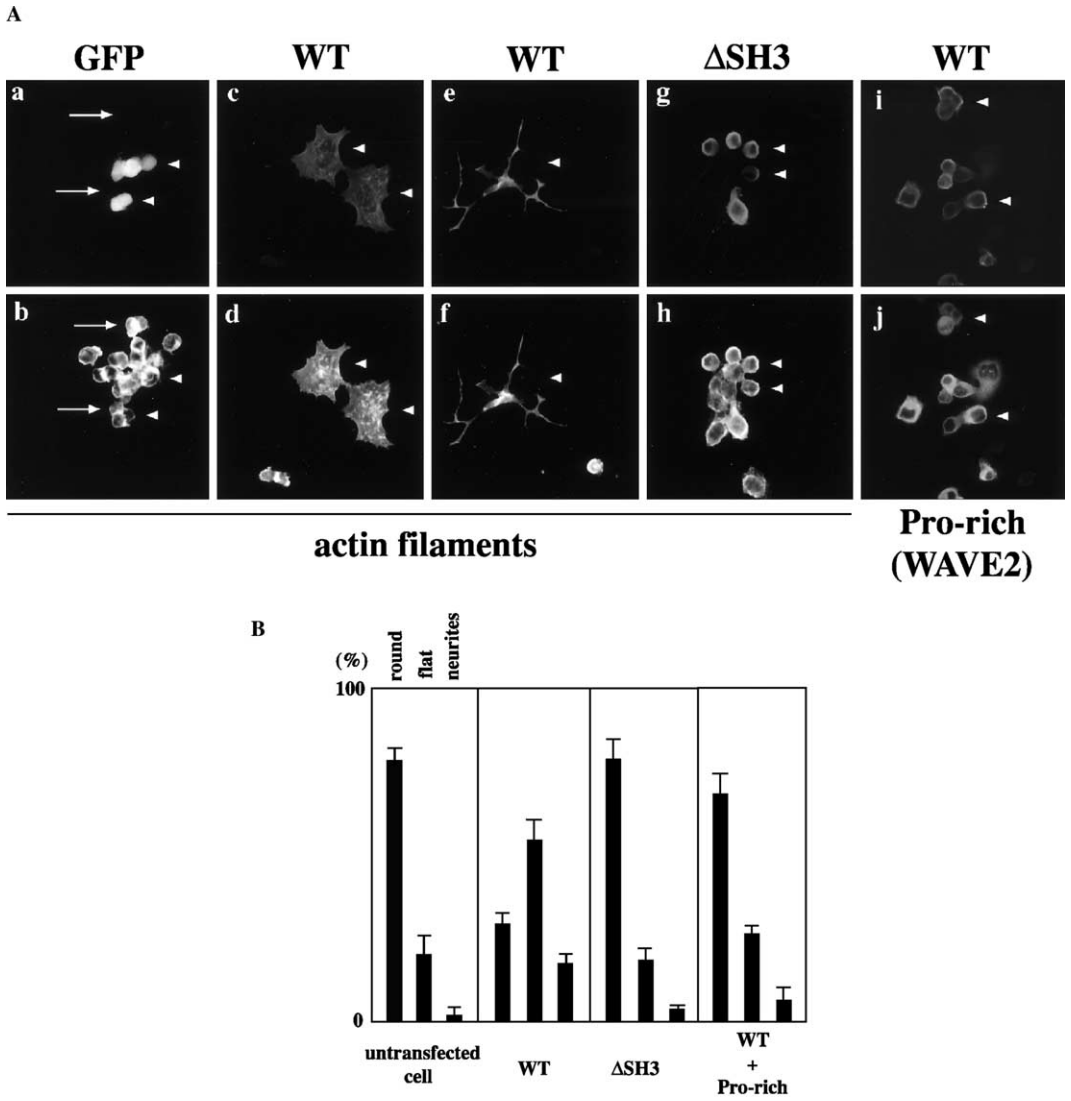

Fig. 4. IRSp53 induces spreading and neurite formation of N1E-115 cells in an SH3 domain-dependent manner. (A) Morphological change induced by IRSp53. N1E-115 cells were transfected with expression plasmids for GFP (a,b), FLAG-tagged WT IRSp53 (c,d,e,f), FLAG-tagged ΔSH3 IRSp53 (g,h), or FLAG-tagged WT IRSp53 with Myc-tagged proline-rich region of WAVE2 (i,j). The signal of GFP (a), phalloidin (b,d,f,h), anti-FLAG antibody (c,e,g,i), and anti-Myc antibody (j) are shown. (a,b) Cells showing rounded morphology. Untransfected cells are indicated by arrows and GFP-expressing cells are indicated by arrowheads. (c,d) Cells showing a typical flattened morphology induced by WT IRSp53 (indicated by arrowheads). (e,f) Cells showing neurite-bearing morphology by WT IRSp53. (g,h) Cells expressing ΔSH3 IRSp53. (i,j) Cells co-expressing WT IRSp53 and the proline-rich region of WAVE2. (B) Quantitation of the effect of IRSp53-expression. Morphologies of the cells were classified as round, flat, or neurites. Round cells are those shown in A(a,b). Flat cells are those shown in A(c,d). The designation neurites was used to classify cells with one or more protrusions with lengths longer than twice the diameter of the cell. The average values obtained from two independent experiments are indicated (50 cells were counted in each experiment). Error bars represent the difference in each experiment from the average values.

morphology. In contrast, the expression of WT IRSp53 induces a significant spreading of the cells and the cells became flattened (Fig. 4A, c,d). We also noticed the occurrence of neurite formation (Fig. 4A, e,f) as previously reported [18]. Quantitative analysis revealed that the flattened morphology was the most distinctive one (54%, Fig. 4B). The neurite-forming rate was only 17%, but it is significant since N1E-115 cells normally do not form neurites in the presence of serum. We then expressed ΔSH3 IRSp53 lacking the SH3 domain. We did not see any significant change in cell morphology, and most of the expressing cells retained a rounded morphology similar to that of parental cells (Fig. 4A, g,h). These results with ΔSH3 IRSp53 differ from those of the previous study [18], in which it was shown to induce neurite formation. One important difference in the experimental procedure is the use of laminin, an extracellular matrix protein. Govind et al. [18] seeded cells on laminin-coated coverslips, whereas we seeded cells directly on uncoated coverslips. Laminin has been shown to stimulate neurite formation in N1E-115 cells [21], which may be the reason for the discrepancy. Our results

suggest the importance of SH3 domain-binding proteins in the IRSp53-induced morphological change. Indeed, when we co-expressed the proline-rich region in WAVE2, which binds strongly to the SH3 domain of IRSp53 [17] and would inhibit the interaction with appropriate SH3 domain-binding proteins, the morphological change was suppressed (Fig. 4A, i,j).

These results suggest the importance of IRSp53 in the regulation of spreading of N1E-115 cells. It was reported that expression of Tiam1, a specific activator for Rac, induces spreading of N1E-115 cells on uncoated coverslips [21]. In this case, activation of Rac by Tiam1 is thought to regulate cellular adhesion via specific subset of integrin receptors. Expression of the activated Rac mutant was also shown to induce spreading of N1E-115 cells [21]. IRSp53 therefore seems to play an important role in cellular spreading by co-operating with Rac.

Mechanism of IRSp53-induced morphological change of N1E-115 cells

There have been several reports of proteins that bind the SH3 domain of IRSp53. These proteins include DRPLA [22], BAI-1 [23], mDia [24], Mena [19], and WAVE2 [17]. Among these binding proteins, at least mDia, Mena, and WAVE2 have established associations with regulation of the actin cytoskeleton and cellular morphologies, and thus they are strong candidates as molecules involved in IRSp53-induced morphological change. One important function of WAVE2 is to activate the Arp2/3 complex and induce rapid actin-polymerization [17,25]. To examine the possible involvement of the Arp2/3 complex in IRSp53-induced morphological change of N1E-115 cells, we co-expressed the CA-fragment of N-WASP, which binds and sequesters the Arp2/3 complex [8,26] with IRSp53. In this case, both cellular spreading and neurite formation were partially suppressed, but the effect was not complete (data not shown). We therefore think that WAVE2 may be important for an IRSp53-induced morphological change of N1E-115 cells, but some other target(s) is(are) also important. A more detailed characterization of binding proteins to the SH3 domain of IRSp53 will be needed to know about the functional mechanism of IRSp53.

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