The RAC Binding Domain/IRSp53-MIM Homology Domain of IRSp53 Induces RAC-dependent Membrane Deformation*

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The concave surface of the crescent-shaped Bin–amphiphysin–Rvs (BAR) domain is postulated to bind to the cell membrane to induce membrane deformation of a specific curvature. The Rac binding (RCB) domain/IRSp53-MIM homology domain (IMD) has a dimeric structure that is similar to the structure of the BAR domain; however, the RCB domain/IMD has a “zeppelin-shaped” dimer. Interestingly, the RCB domain/IMD of IRSp53 possesses Rac binding, membrane binding, and actin filament binding abilities. Here we report that the RCB domain/IMD of IRSp53 induces membrane deformation independent of the actin filaments in a Rac-dependent manner. In contrast to the BAR domain, the RCB domain/IMD did not cause long tubulation of the artificial liposomes; however, the Rac binding domain caused the formation of small buds on the liposomal surface. When expressed in cells, the Rac binding domain induced outward protrusion of the plasma membrane in a direction opposite to that induced by the BAR domain. Mapping of the amino acids responsible for membrane deformation suggests that the concave surface of the Rac binding domain binds to the membrane in a Rac-dependent manner, which may explain the mechanism of the membrane deformation induced by the RCB domain/IMD.

IRSp53 is an adaptor protein with an N-terminal Rac binding (RCB) domain (residues 1–228) and an Src homology 3 domain. RNA interference of IRSp53 is reported to decrease lamellipodia formation (1). However, membrane protrusion with very dim or absent phalloidin (a reagent that stains actin filament) staining has been observed in the IRSp53-overexpressing cells (2, 3). This observation suggested that IRSp53 might affect membrane organization independent of the actin filaments.

The RCB domain of IRSp53 shares its homology with the MIM (missing in metastasis) protein, and this domain is also named the IRSp53/MIM homology domain (IMD) (residues 1–250 of IRSp53). Activated Rac binds to the RCB domain of IRSp53 (4, 5). In contrast, the IMD of MIM is reported to bind to the inactive form of Rac (6).

In addition to the Rac binding (RCB) activity, the RCB domain/IMD of IRSp53 or MIM possesses the actin filament bundling activity (7). Overexpression of IMD is shown to cause actin filament-containing protrusions; however, some of the protrusions were branched and lacked the phalloidin staining (7).

Recently, Millard et al. (8) reported the crystal structure of the IMD fragment (residues 1–250); this fragment is a “zeppelin-shaped” dimer of a bundle of three extended α-helices and a shorter C-terminal helix. They found that the basic residues at the extreme ends of the dimer are important for the actin filament bundling activity. The substitution of the basic residues with Glu is reported to reduce the actin binding activity by 50%.

The IMD structure suggested that the bundle of three extended α-helices is structurally similar to the Bin/amphiphysin–Rvs (BAR) domain. This domain has a curved structure, and its concave surface is postulated to bind to the cell membrane. The expressed amphiphysin BAR domain was targeted to intracellular vesicles, and it caused membrane tubulation (9–11). The purified BAR domain protein induced liposome tubulation in vitro (9). Thus, the BAR domain is a membrane-binding and tubulation module. In the BAR domain, the concave surface and the basic residues at the extreme ends are involved in the binding of the BAR domain to the membrane.

Therefore, the structural similarity and presence of protrusions without the actin filaments suggest that RCB domain/IMD is a membrane-binding module as well as an actin filament-binding module.

In this study, the crystal structure of the RCB domain (resi-
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dues 1–228) was determined; the three-helix bundle dimer was formed in the absence of helix 4 of the IMD (residues 1–250), demonstrating that the overall structure of the RCB domain/IMD was similar to that of the BAR domain. The RCB domain expression in the cells caused membrane protrusions that lacked phalloidin staining in a Rac-dependent manner. Moreover, in the presence of Rac, the isolated RCB domain induced deformation of the artificial liposomes in a manner that was different from that induced by the BAR domain. Mapping of the residues responsible for membrane deformation suggests the involvement of the convex surface of the RCB domain in the binding of the RCB domain to the membrane. These findings indicate that the RCB domain is a Rac-dependent membrane tubulation module as well as an actin filament-bundling module.

**EXPERIMENTAL PROCEDURES**

**Purification**—The human IRSp53-RCB domain (residues 1–228) was expressed as an N-terminal GST fusion protein in *Escherichia coli* BL21 (DE3) cells. The fragment encoding the RCB domain was excised from full-length IRSp53 by BamHI and SmaI digestions. This fragment was inserted into the BamHI and SmaI sites of pGEX 4T-1 (Amersham Biosciences). The BamHI site was followed by ACC (Thr) and then the first amino acid residue. The protein was purified to 54.6 mg/ml in 20 mM Hepes buffer, pH 7.6, 0.1 mM EDTA, 1 mM MgCl₂, 100 mM KCl, and 2 mM dithiothreitol. The final sample included genetically manipulated fragments (residues 2–0 and 229–239). IRSp53 and Rac were expressed in a baculovirus system and purified as an N-terminal GST fusion protein.

**Crystallization and Data Collection**—The IRSp53-RCB domain was crystallized by the hanging drop vapor diffusion method. The protein solution (8.7 mg/ml) was mixed with an equal volume of reservoir solution, including 90 mM Tris-HCl buffer, pH 8.5, 27% PEG4000, 190 mM sodium acetate, and 3.5% 2-methyl-2,4-pentanediol. A mercury derivative was obtained by soaking the crystals in 5 mM ethylmercurithiosalicylic acid in 2-methyl-2,4-pentanediol. A mercury derivative was obtained. The crystal was grown by soaking the crystals in 5 mM ethylmercurithiosalicylic acid in 2-methyl-2,4-pentanediol. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown. The crystal was grown.

**Completeness (last shell), %**

- Native I: 98.6 (98.2)
- Native II: 98.5 (97.6)
- Hg derivative: 99.2 (98.6)

**Resolution range, Å**

- Native I: 50 to 2.63
- Native II: 50 to 3.00
- Hg derivative: 1950.3.20

**Wavelength, Å**

- Native I: 1.5418
- Native II: 1.5418
- Hg derivative: 1.5418

**No. of references**

- Native I: 66,002
- Native II: 40,479
- Hg derivative: 66,009

**No. of unique references**

- Native I: 15,858
- Native II: 10,720
- Hg derivative: 8937

**Completeness (last shell), %**

- Native I: 98.6 (98.2)
- Native II: 98.5 (97.6)
- Hg derivative: 99.2 (98.6)

**Rsym* (last shell), %**

- Native I: 5.9 (30.3)
- Native II: 8.0 (27.2)
- Hg derivative: 11.7 (33.8)

**I/I(0)**

- Native I: 26.1 (5.5)
- Native II: 16.4 (5.4)
- Hg derivative: 16.8 (8.4)

**Resolution range, Å**

- Native I: 41.9 to 2.63
- Native II: 34.5 to 2.63
- Hg derivative: 1950.3.20

**No. of protein atoms**

- Native I: 3644
- Native II: 3644
- Hg derivative: 3644

**No. of water molecules**

- Native I: 70
- Native II: 70
- Hg derivative: 70

**Bonds, °**

- Native I: 0.007
- Native II: 23.5; 29.7
- Hg derivative: 1.1

**Angle, Å**

- Native I: 1.1
- Native II: 1.1
- Hg derivative: 51

The calculated electron density was of sufficient quality to trace the main chains. The model was built manually by O (14) and was refined with the use of the CNS program package (15). All refinement steps were monitored with the free R-factor, based on 10% of the x-ray data. After a simulated annealing protocol, the structure was refined by atom-positional and temperature factor refinement, as well as manually. The figures were produced with PyMol. Surface electrostatic potentials were prepared with GRASP (16).

**ELISA Lipid Binding Assay**—Lipid vesicles (phosphatidylethanolamine (PE)/phosphatidylcholine (PC) = 1:1, total 2 μg), containing 10% (by weight) of phosphatidylinositol triphosphate (PIP₃) resuspended in 100% ethanol were coated onto 96-well plates (Immulon 2HB; Thermo Labsystems) and air-dried at room temperature. The wells were then blocked with 5% bovine serum albumin in phosphate-buffered saline, before incubation with the appropriate glutathione S-transferase (GST) fusion protein (1.0 μg ml⁻¹) for 45 min. The wells were washed with 0.05% Tween 20 in phosphate-buffered saline, and glutathione-conjugated peroxidase (1.0 μg ml⁻¹, Sigma) was overlaid and detected by a reaction with ortho-phenylenediamine. Absorbance was measured at 492 nm with an ELISA plate reader (Bio-Rad).

**Pulldown Assay for Rac-RCB Domain Association**—Rac protein was purified from *E. coli*, and was loaded with GTPγS as described previously (17). RCB or IMD domain was expressed as an N-terminal GST fusion protein. Equal amounts of various RCB or IMD GST fusion proteins were immobilized on beads, and binding to GTPγS-loaded Rac (2 μM) was examined. After washing, bound Rac was analyzed by Western blotting and densitometry. The amount of bound Rac was expressed as arbitrary units.

**Actin Filament Sedimentation Assay**—Binding of filamentous (F)-actin to the RCB domain of IRSp53 was analyzed by co-sedimentation assay. A globular (G)-actin solution was first clarified by ultracentrifugation at 100,000 × g for 20 min, and

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**TABLE 1**

| Data collection and refinement statistics | Native I | Native II | Hg derivative |
|------------------------------------------|---------|----------|---------------|
| Space group                              | P₂₁     | P₂₁      | P₂₁           |
| Cell dimensions, Å                       |         |          |               |
| a                                        | 60.833  | 60.845   | 60.743        |
| b                                        | 69.258  | 69.783   | 69.635        |
| c                                        | 68.715  | 67.954   | 67.997        |
| β                                        | 110.124 | 110.183  | 109.967       |
| Resolution range, Å                      | 50 to 2.63 | 50 to 3.00 | 1950.3.20 |
| Wavelength, Å                            | 1       | 1.5418   | 1.5418        |
| No. of references                        | 66,002  | 40,479   | 66,009        |
| No. of unique references                 | 15,858  | 10,720   | 8937          |
| Completeness (last shell), %             | 98.6 (98.2) | 98.5 (97.6) | 99.2 (98.6) |
| Rsym* (last shell), %                    | 5.9 (30.3) | 8.0 (27.2) | 11.7 (33.8) |
| I/I(0)                                   | 26.1 (5.5) | 16.4 (5.4) | 16.8 (8.4) |
| Resolution range, Å                      | 41.9 to 2.63 | 34.5 to 2.63 | 1950.3.20 |
| No. of protein atoms                     | 3644    | 3644     | 3644          |
| No. of water molecules                   | 70      | 70       | 70            |
| Root mean square deviation               |         |          |               |
| Bonds, °                                 | 0.007   | 23.5; 29.7 | 1.1          |
| Angle, Å                                 | 1.1     | 1.1      | 51            |

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*(Sym* = Σ|Fo| - |Fc|)/Σ|Fo|, where |Fo| and |Fc| are the observed and calculated structure factor amplitudes.)
the G-actin was polymerized to F-actin for 1 h at room temperature. Various GST-RCB domain solutions (2 μM in 10 mM Hepes, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 5 mM EGTA, and 0.1 mM CaCl₂) were also clarified by ultracentrifugation. F-actin (2 μM) and RCB domain solutions were mixed and incubated at room temperature for 1 h, and the mixture was centrifuged at 100,000 g for 20 min. Supernatants and pellets (equal volumes) were then analyzed by SDS-PAGE followed by densitometry. Percentages of protein amount in the precipitates are shown with standard deviations.

Liposome Co-sedimentation Assay—Synthetic liposomes contained PC/PE/PIP₃ (weight ratio) or PC/PE/PS/PIP₃ in 50 mM Hepes, pH 7.9, 150 mM NaCl and were subjected to five cycles of freezing (liquid N₂) and thawing (37 °C water bath). RCB and/or Rac, at the indicated concentrations, and liposomes (0.5 mM for PC) were mixed in the same buffer (association phase). Binding was examined at five or more different protein concentrations, and K₅₀ (k_D/k_A) values were calculated from curve fitting. The observed association rates (k_on) over the initial 60 s of association were plotted (Fig. 4C). The slope and intercept yielded k_a and k_d values, respectively.

Electron Microscopy—RCB domain was expressed as an N-terminal GST fusion protein in E. coli. IRSp53 and Rac proteins were expressed in a baculovirus system and were purified as described previously (1). Electron microscopy was performed as described previously (18, 19). Liposomes (Folch fraction 1; Sigma) (0.1 mg/ml final concentration) were incubated at 37 °C for 15 min in buffer XB (10 mM Hepes, pH 7.9, 100 mM KCl, 5 mM EGTA, 0.1 mM CaCl₂, 2 mM MgCl₂) with RCB domain or IRSp53 (0.5 μM) and GTPγS-loaded Rac (0.5 μM). At the end of the incubation, aliquots were adsorbed onto collodium-coated copper electron microscopy grids (Nissin EM) for 1 min at room temperature, washed in 0.1M Hepes, pH 7.4, stained in 1–2% uranyl acetate for 30 s, blotted, and then allowed to air dry.

Time-lapse Microscopy of Liposome Deformation—Time-lapse analysis of liposome deformation was performed as described previously (20). To adhere liposomes onto glass coverslips, two 1-μl droplets of lipid solution (10 mg/ml Folch fraction 1 (Sigma) in chloroform) were spotted onto each coverslip and allowed to dry under vacuum for at least 1 h. Lipids were then prehydrated in 50 μl of XB + 1 mg/ml bovine serum albumin for 20–30 min at 37 °C in a small chamber built by placing the coverslip over a glass slide with strips of double-sided tape as spacers. Twenty microliters of protein solution (1 mg/ml) was then injected between the glass pieces. Time-lapse images were analyzed using ImageJ software.

**FIGURE 1.** Amino acid sequence alignment among the RCB domains and IMDs. Identical and highly conserved residues are shown in black. Conserved residues among IRSp53/IRTKS/FLJ22582 are shown in gray. Columns indicate helices in the RCB domain. The residues for the binding of the RCB domain to lipids, Rac, and actin filaments are labeled by their amino acid numbers.
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A
RCB domain

B
Front view
RCB (IRS5p53)

BAR (Arfaptin)

BAR (Amphiphasin)

C

D

Front view

Left
Right

K147
K130
K171

K108
K147

E

F

Front view

Left
Right

K147
K130
K171

K147
K143

G

H

Front view

Left
Right

K147
K142
K147

K147
K142

% of precipitated RCB protein

ppholos

p < 0.001

p < 0.05

p < 0.001

p < 0.05

"
were obtained with a phase contrast microscope (Zeiss) equipped with a camera (CCD-782-Y/HS; Princeton Instruments). ×40 oil-immersion objective (NA = 1.30 FLUAR; Zeiss) was used.

Cell Culture, Transfection, and Microscopy—COS-7 cells were cultured as described previously (21). Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. RCB domain was tagged N-terminally with a brighter variant of GFP (Venus) (22) and was expressed under the control of the human cytomegalovirus immediate early promoter. DNA encoding constitutively active Rac (G12V mutant) was inserted into the pEF-BOS vector with a Myc tag (4). After fixation, permeabilization, and blocking of cells, cells were stained with an anti-GFP antibody (MBL) or anti-Myc antibody (9E10; Sigma) followed by an Alexa-488- or Alexa-633-conjugated secondary antibody (Molecular Probes). Actin filaments were visualized by rhodamine-conjugated phalloidin (Molecular Probes). Latrunculin B (1 μg/ml) was treated for 30 min before fixation. For membrane staining, membrane-staining dye (DiI (DiIC18(3)); Molecular Probes) was incubated with cells before fixation, and then cells were observed without permeabilization and antibody staining. Fluorescent images were taken through a microscope (Nikon) with a confocal microscopy system (Radience 2000; Bio-Rad) at room temperature. ×60 oil immersion objective NA = 1.40 (Nikon) was used. Images were assembled with Adobe Photoshop. In each plate, photographs were cropped, and each fluorochrome was adjusted identically for brightness and contrast to represent the observed images. Time-lapse analysis was performed with total-internal-reflection microscopy system (Olympus) with ×100 oil immersion objective NA = 1.45 (Olympus) and MetaMorph software.

Coordinates—Atomic coordinates of the IRSp53 RCB domain have been deposited in the Protein Data Bank (accession code 1WDZ).

**RESULTS AND DISCUSSION**

**Structure of the RCB Domain**—The three-dimensional structure of the RCB domain (amino acid residues 1–228) (Fig. 1) of IRSp53 was determined by single isomorphous replacement with the anomalous scattering method. The RCB domain structure had an asymmetric unit that included two subunits, A and B; this unit formed an elongated homodimer with a length of ~180 Å (Fig. 2A). With the exception of the subunit B residues 150–158, which could not be identified because of a disorder, polypeptide chains were traced for both the subunits in an electron density map. The subunits were related by a local 2-fold rotation axis and were superimposed with a root mean square deviation of 0.497 Å (mean value for the 222 common C-α atoms). Each subunit consisted of six α-helices, namely α1–α6 (Fig. 1), that are arranged as an antiparallel helix bundle. The helix bundle structure of the IRSp53 RCB domain was almost identical to the corresponding portion of the reported structure of the IRSp53 IMD, which is 22 residues longer than the RCB domain (8). These additional IMD residues form a short helix (helix 4) that interacts with the other three helices in the IMD structure (8). Despite the absence of this short helix, the RCB domain formed a bundle structure, suggesting that the short helix does not contribute to bundle formation. Thus, the RCB domain is an independent entity with a structure and surface electrostatic properties similar to that of the BAR domain (Fig. 1 and Fig. 2, A and B).

**Membrane-binding Sites of the RCB Domain**—The RCB domain and full-length IRSp53 bind to lipids presumably via electrostatic interactions with little selectivity for specific phosphoinositides (1). To determine the lipid-binding surface of the RCB domain dimer, we constructed a series of Glu and/or Ala substitutions for 20 Lys or Arg residues and tested its lipid binding ability by ELISA (Fig. 2C) (23). At the ends of the dimer, there is a basic patch consisting of Lys-142, Lys-143, Arg-145, Lys-146, and Lys-147. A basic patch mutant (BPM), in which all these five residues were replaced by Ala, did not bind to negatively charged phosphoinositides (Fig. 2C); the structure of the RCB domain was not destroyed, as revealed by the circular dichroism spectrum (data not shown). Next, we replaced two of the basic amino acids in the basic patch according to their side chain orientations. The replacement of Lys-142 and Arg-145 by either Ala (K142A/R145A) or Glu (K142E/R145E) did not affect the RCB domain-lipid binding. In contrast, the K143A/K147A and K143E/K147E mutations dramatically reduced the RCB domain-lipid binding (Fig. 2C), whereas the K143E mutation did not affect this binding. Therefore, Lys-147 was determined to be the most important residue in the basic patch.

Arg-128 and Lys-130 are located close to each other near the basic patch. The RCB domain-lipid binding was significantly decreased by the R128E/K130E mutations. Furthermore, the K108E and K171A mutants showed decreased lipid binding, whereas Glu substitutions for Arg-29, Lys-40, Lys-70, Lys-121, Lys-136, Lys-152, Lys-156, Lys-160, and Arg-192 did not (Fig. 2C). Therefore, the RCB domain-lipid binding involves a limited region; among the basic residues tested, Lys-108 (α4), Lys-130 (α5), Lys-147 (α5–α6 loop), and Lys-171 (α6) were determined to be particularly important for this binding (Fig. 1 and Fig. 2D). With the exception of Lys-147, these Lys residues are located in a straight line along the straight helix bundle (Fig. 2D, front view). Lys-147 is located on the flexible α5–α6 loop and can relocate its side chain to position itself on

![FIGURE 2. Mapping of the RCB domain residues responsible for binding of the RCB domain to lipids, Rac, and actin filaments. A, ribbon diagrams of the RCB domain and the IMD. Monomers A and B are colored cyan and light green, respectively. Helix 4 in the IMD is shown in orange. Black ellipse and line represent the local 2-fold symmetry. B, surface electrostatic potentials of the RCB domain and the arfaptin and amphiphysin BAR domains. Positively charged regions are shown in blue, and negatively charged regions are shown in red. C, lipid binding ability of various RCB domain mutants as determined by ELISA. E, Rac binding ability of various mutants of the RCB domain and the MIM IMD domain. G, actin filament binding ability of various RCB domain mutants. Error bars represent the S.D. of at least three independent experiments. Statistical significance was analyzed by Student’s t test. D, F, and H, molecular surface representations of the RCB domain of IRSp53. Amino acids used for the mutation analysis of the RCB domain-lipid (D), RCB domain-Rac (F), and RCB domain-actin binding activities (H) are shown in red (reduced activity) and green (no change). Each figure includes a front view (perpendicular to the noncrystallographic 2-fold axis) and a side view (along the noncrystallographic 2-fold axis).
the straight line (Fig. 1 and Fig. 2D). Each protomer of the dimeric helix bundle possesses this “lipid-binding line”; the two lines are located symmetrically on opposite sides of the dimer. In the “front” view of the helix bundle (Fig. 2D, upper panel), the lipid-binding line on the right is visible, whereas that on the left is located on the opposite side of the dimer (invisible in this panel). If the dimer is sufficiently stable and does not bend, only one of the lipid-binding lines is used when the dimer binds to the membrane surface.

Interestingly, the corresponding region of MIM (1–238 amino acids) had less lipid binding ability (Fig. 2C). Consistently, the Lys-108, Lys-147, and Lys-171 are occupied with neutral amino acids in MIM (Fig. 1).

Based on the binding curve, the $K_d$ value between the RCB domain and phosphatidylserine (PS)-containing liposome was estimated to be $\sim 3 \mu M$ (Fig. 3A).

**Rac-binding Sites of the RCB Domain**—In contrast to the binding of IRSp53 to the GTP-loaded Rac, MIM was reported to bind to the GDP-loaded Rac (4, 6). Therefore, we performed pulldown assays to test the Rac binding ability of the RCB domain mutants (Q23E, R11E/Q23E, K40E, K58E, K70E, K121E, R128E/K130E, K142E/R145E, K143E, K143E/K147E, and BPM), in which the conserved residues (underlined) or the nonconserved residues between IRSp53 and MIM were replaced (Fig. 1). Arg-11 was considered to contribute to Rac binding because the RCB domain mutations of this residue...
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The R11E/Q23E mutant of RCB was independent of the order of the incubation of Rac and liposome. However, the lip-id-binding defective mutant of RCB (R128E/K130E) had increased affinity to liposome when the R128E/K130E mutant was incubated with Rac prior to addition of liposome (Fig. 3C). In contrast, when the R128E/K130E mutant was incubated with liposome prior to the Rac addition (simultaneous exposures of Rac to the RCB mutant and liposome), no increase of the affinity in the R128E/K130E binding to liposome was observed (Fig. 3C). These results suggested that the binding of membrane to the RCB domain is required for the association between RCB domain and Rac on the membrane.

Rac purified from E. coli did not show lipid modification, and it decreased the binding of the RCB domain to the membrane compared with Rac purified from the Sf9 cells, indicating that the lipid modification of Rac is required for simultaneous binding of RCB domain to lipids and Rac (Fig. 3D).

Therefore, Rac and lipid slightly compete with each other for binding to RCB domain. However, Rac with lipid modification appears to bind to the membrane-bound RCB domain, presumably affecting the mode of binding of the RCB domain to the membrane.

Actin Filament-binding Sites of the RCB Domain—The IMDs of IRSp53 and MIM mediate actin filament bundling (7), and the bundling activity of the IRSp53 IMD is decreased by 50% because of simultaneous Glu substitutions for Lys-142, Lys-143, Lys-146, and Lys-147 in the basic patch (8). The shorter

(8) The R128E/K130E mutant showed normal binding to actin filaments (Fig. 2C).

To study the RCB domain-actin filament binding, we tested a series of basic residue mutants (Q23E, R11E/Q23E, K108E, R128E/K130E, K130E, K136E, K142E/R145E, K143E, and K143E/K147E), including mutants with decreased lipid binding and/or Rac binding abilities. The K142E/R145E mutant showed normal RCB domain-lipid binding (Fig. 2C) but decreased RCB domain-actin filament binding (Fig. 2G).

Lys-142 and Arg-145 were conserved between IRSp53 and MIM (Fig. 1). In contrast, the R128E/K130E mutant showed normal binding to actin filaments despite decreased binding to lipids (Fig. 2, C and D). The K143E/K147E mutant, not the K143E mutant, showed...
decreased binding to the actin filament and lipids (Fig. 2, C and G), suggesting that Lys-147 is important for binding to both actin filaments and lipids. Thus, actin filaments bind to the basic patch (Lys-142, Arg-145, and Lys-147) at the end of the RCB domain, whereas lipids bind to the lipid-binding line, which contained the basic residues (Fig. 2, D and H). Mutations in the Rac-binding site (R11E/Q23E and K143E) did not affect its binding to the actin filament (Fig. 2G).

Residues for Binding of the RCB Domain to Membrane, Rac, and Actin Filaments Are Involved in the RCB Domain-induced Deformation of Artificial Liposomes in Vitro—We next examined the shapes of liposomes after their incubation with the purified RCB domain or full-length IRSp53 in the presence or absence of Rac. After incubation of the RCB domain with liposomes, a small number of bud-like liposomes formed on the surface of relatively large liposomes (Fig. 4A). When liposomes were incubated with the GTPγS-loaded Rac and the RCB domain, clusters of bud-like liposomes were formed presumably on the existing liposomes (Fig. 4A). Budding was not observed in response to incubation with Rac alone. As a control, the BAR domain of Bin 1/amphiphysin 2 was incubated with liposomes, and tubulation was observed, as reported previously (Fig. 4A) (9). In addition, we observed the Rac-dependent formation of clusters of bud-like liposomes in response to incubation of liposomes with full-length IRSp53 (Fig. 4B).

We then tested whether the RCB domain mutants could induce liposomes to form clusters of buds in the presence of Rac. As expected, the lipid binding-deficient R128E/K130E mutant showed no ability to induce the formation of small buds on the liposomes (Fig. 4C). Consistent with the Rac-dependent formation of small buds on the liposomes by the wild-type RCB, the Rac binding-deficient R11E/Q23E or K143E mutants showed greatly decreased ability to induce the formation of small buds on the liposomes (Fig. 4C).

Most surprisingly, the actin filament binding-deficient K142E/R145E mutant showed decreased formation of small buds on the liposomes, indicating that residues involved in RCB domain-actin filament binding contribute to membrane deformation after the RCB domain binds to liposomes (Fig. 4C).
Possible Membrane Deformation Induced by the RCB Domain in a Direction Opposite to That Induced by the BAR or EFC/F-BAR Domains—The deformation of the liposomes to form clusters of small buds indicates two possible mechanisms of membrane deformation. One is the process of tubulation induced by the RCB domain in a manner similar to that induced by the BAR or EFC/F-BAR domains. The other is membrane deformation induced by the RCB domain in a direction oppo-
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A site to that induced by the BAR domain. In the latter case, compression of the spherical liposomes is thought to result in the budding of excess lipid bilayer by protrusion of the materials present inside the liposomes. Therefore, we analyzed the shape of large liposomes in the presence of the RCB protein and Rac using light microscopy. Incubation of liposomes with the EFC/F-BAR domain was performed as a control, and it resulted in the formation of long and thin protrusions from the edges of the large liposomes, as reported previously (20, 27) (Fig. 4E). When the large liposomes generated onto glass coverslips were incubated with the RCB domain and Rac, regression of the edges of the liposomes was observed (Fig. 4D). Eventually, budding at the edges of the large liposomes was observed. Long and thin protrusions were never observed when large liposomes were incubated with the RCB domain and Rac.

Protrusions Induced by Expression of the RCB Domain in Cells—When the RCB domain fragment was tagged with GFP and expressed in cells, we observed extensive microspike-like structures of GFP-RCB localization (Fig. 5). As reported for full-length IRSp53 (2, 3), formation of microspike-like structures without phalloidin staining was observed in the COS-7 and A431 cells as well as in the mouse embryonic fibroblasts (Fig. 5). The protrusions were branched; however, these were not because of the retraction of the cells, as revealed by the time-lapse recording (Fig. 6). Furthermore, these structures appeared to lack the actin filaments, because phalloidin staining was almost absent even after the signals were highly intensified in most of the protrusions (Fig. 5). Therefore, these structures were formed even after a 30-min treatment with latrunculin B, a drug that inhibits actin polymerization (Fig. 5). Therefore, these structures are considered to represent protrusion of the plasma membrane.

To address the Rac dependence of the RCB domain-induced plasma membrane protrusion, we co-expressed a dominant-negative mutant of Rac (T17N) with the wild-type RCB domain in the COS-7 cells. Although some protrusions with phalloidin staining remained, most of the protrusions were significantly decreased in the cells expressing Rac T17N and the RCB domain (Fig. 5).

Next, we tested various RCB mutants for their ability to induce protrusions. Mutants deficient in the Rac binding ability (R11E/Q23E or K143E) or the MIM-(1–228) fragment did not induce membrane protrusion without phalloidin staining in the COS-7 cells (Fig. 7, A, B, and E). Instead, smaller microspikes that contained actin filaments were formed. These results indi-
cate that binding of the RCB domain to activated Rac is essential for the extensive protrusion of the plasma membrane.

We next expressed the RCB domain mutants with reduced affinities for lipid and/or actin in cells. Protrusions were not induced in the cells expressing the R128E/K130E mutant, which possesses decreased lipid binding ability but normal Rac and actin filament binding abilities, or the K142E/R145E mutant, which possesses decreased actin filament binding ability but normal lipid and Rac binding abilities (Fig. 7, A, B, and E). Thus, all the residues that are required for the binding of the RCB domain to Rac, membrane, and actin filaments are required for causing membrane protrusions in cells.

To confirm these protrusions contain cell membrane, cells were stained with the membrane-staining dye (DiI). The protrusions containing RCB proteins were stained by DiI (Fig. 7, C and D). A small number of protrusions stained by DiI did not contain RCB proteins in mutant RCB-expressing cells (Fig. 7, C and D, and data not shown), indicating the specific localization of wild-type RCB domain protein in RCB-induced protrusions.

Possible Mechanism of Membrane Deformation Induced by the RCB Domain—The shape of the liposomes in vitro did not directly explain the shape of cell protrusions. Therefore, it remains unclear whether the protrusion of cells was the direct consequence of the membrane deformation observed in vitro. Furthermore, we cannot completely rule out the involvement of actin cytoskeleton in the RCB-induced protrusions because the amount of actin filaments may be too small to be detected by actin cytoskeleton in the RCB-induced protrusions because the close proximity of these two sites. Interestingly, the actin filament-binding site on the left that faces the membrane appears to bind to the membrane by the electrostatic interaction if the association of the RCB domain with Rac fixes the binding of the lipid-binding site on the right to the membrane (Fig. 2H and Fig. 8A). Slight overlap of Rac and the lipid-binding line may alter the orientation of the RCB domain toward the membrane to cause the membrane deformation when Rac is associated with the RCB domain. Thus, the lipid-binding line and the actin filament-binding site on the RCB domain appear to present the convex surface of the RCB domain for binding this domain to the membrane in the presence of Rac (Fig. 8A).

It is postulated that if the tubules that are formed by inward membrane tubulation are surrounded by the concave surface of the BAR domain (Fig. 8B), the tubules formed by the outward membrane tubulation are lined by the convex surface of the RCB domain (Fig. 8C). The detailed analysis of the binding of the RCB domain to the membrane is required for understanding the mechanism of the inward and outward membrane protrusion.

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