REV7 has a dynamic adaptor region to accommodate small GTPase RAN/Shigella IpaB ligands, and its activity is regulated by the RanGTP/GDP switch

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REV7, also termed mitotic arrest–deficient 2-like 2 (MAD2L2 or MAD2B), acts as an interaction module in a broad array of cellular pathways, including translesion DNA synthesis, cell cycle control, and nonhomologous end joining. Numerous REV7 binding partners have been identified, including the human small GTPase Ras-associated nuclear protein (RAN), which acts as a potential upstream regulator of REV7. Notably, the Shigella invasin IpaB hijacks REV7 to disrupt cell cycle control to prevent intestinal epithelial cell renewal and facilitate bacterial colonization. However, the structural details of the REV7–RAN and REV7–IpaB interactions are mostly unknown. Here, using fusion protein and rigid maltose-binding protein tagging strategies, we determined the crystal structures of these two complexes at 2.00–2.35 Å resolutions. The structures revealed that both RAN and IpaB fragments bind the “safety belt” region of REV7, inducing rearrangement of the C-terminal β-sheet region of REV7, conserved among REV7-related complexes. Of note, the REV7-binding motifs of RAN and IpaB each displayed some unique interactions with REV7 despite sharing consensus residues. Structural alignments revealed that REV7 has an adaptor region within the safety belt region that can rearrange secondary structures to fit a variety of different ligands. Our structural and biochemical results further indicated that REV7 preferentially binds GTP-bound RAN, implying that a GTP/GDP-bound transition of RAN may serve as the molecular switch that controls REV7’s activity. These results provide insights into the regulatory mechanism of REV7 in cell cycle control, which may help with the development of small-molecule inhibitors that target REV7 activity.

When a DNA lesion stalls the replication fork and pauses normal DNA synthesis, REV1 is recruited by ubiquitinated proliferating cell nuclear antigen to the DNA lesion site (5–8). REV7 directly interacts with both REV1 and REV3 (the catalytic subunit of DNA polymerase ζ), which brings TLS polymerases together to overcome the DNA lesions (3, 9–11). In addition to its TLS function, REV7 is the paralog of the human spindle assembly checkpoint protein MAD2, playing important roles in cell cycle regulation. REV7 inhibits the anaphase-promoting complex (APC/C) by directly binding CDH1, an activator of APC/C, to modulate the metaphase-to-anaphase transition (12–14). Recent studies also highlighted a novel role of REV7 in DNA double-strand break repair. REV7 and the other three subunits form one quaternary complex named “Shieldin”, which protects the single-strand DNA ends, antagonizes BRCA1-dependent homologous recombination, and promotes nonhomologous end joining (15–19). Taken together, these studies suggest the importance of REV7 as a regulating interaction module in a variety of cellular pathways.

Previous structural studies have primarily focused on the REV7–3–1 complex in TLS (4, 9–11, 20), and only recently has attention turned to related study of the REV7–CAMP complex in mitosis (21). Structures indicated that REV3 and CAMP share a consensus REV7-binding motif (RBM) with REV3 to bind the same “safety belt” region of REV7 (21). Moreover, REV7 has become a drug target for developing small-molecule inhibitors to disrupt mutagenic translesion synthesis to enhance chemotherapy (22–25). Future structural characterization of REV7-related complexes would help us to understand the interactor-recognizing mechanism of REV7 and open new opportunities for drug discovery.

As the small GTPase Ras-associated nuclear protein (RAN) is a potential upstream regulator of REV7, we focused on structural study of the REV7–RAN interaction. RAN has weak GTP hydrolysis activity and adopts two forms: a GTP-bound form (RanGTP) and a GDP-bound form (RanGDP). During mitosis, RanGTP is generated on chromosomes, establishing a field of RanGTP around mitotic chromosomes (26, 27). RanGTP and RanGDP adopt different conformations involving the C-terminal extension of RAN, leading to different affinity between RAN phosphoprotein; RBM, REV7-binding motif; RAN, Ras-associated nuclear protein; co-IP, coimmunoprecipitation; SPR, surface plasmon resonance; TCEP, tris(2-carboxyethyl)phosphine; CBD, chaperone-binding domain.

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4 The abbreviations used are: TLS, translesion DNA synthesis; APC/C, anaphase-promoting complex; CAMP, chromosome alignment–maintaining

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and RAN-binding effectors (28–32). Through careful regulation of the GTP/GDP cycle, RAN serves as a molecular switch by interacting with RAN-binding proteins to control a broad array of fundamental cellular processes, including nuclear transport, mitotic spindle assembly, and nuclear envelope formation (32). The physical interaction of REV7 with RAN is consistent with the putative role of REV7 in spindle assembly and chromosome alignment. However, the mechanism of the REV7–RAN complex is mostly unknown.

Another REV7 binding partner drawing our attention is *Shigella* Invasin IpaB. IpaB is secreted at the tip of the T3SS needle, forming a membrane pore–forming complex on the host cell membrane to mediate translocation of effectors into the host cell (33). In this process, IpaB also interacts with lipid raft–associated proteins, such as CD44, on the epithelial cell membrane to promote invasion of epithelial cells (34–36). On the other hand, IpaB protein delivered into macrophages can activate caspase-1 and induce apoptosis of macrophages to help *Shigella* escape from immune cells (37–39). Notably, IpaB can target REV7 to interfere with the cell cycle of the host cell and lead to cell cycle arrest at the G2/M phase, which prevents self-renewal of intestinal epithelial cells and facilitates colonization with bacteria (40). Up to now, *Shigella* Invasin IpaB is the only identified pathogen effector to directly target REV7, but the molecular mechanism is still exclusive.

In this study, we determined the crystal structure of the human REV7–RAN fusion protein complex and the crystal structure of the rigid MBP-tagged REV7–IpaB fusion protein complex. Upon RAN or IpaB fragment binding to the safety belt region of REV7, REV7 transforms from the open to the closed conformation. A detailed interaction analysis explains the consensus residues but also sequence-dependent uniqueness of different RBMs. Remarkably, structural alignment reveals a dynamic adaptor region within the safety belt region of REV7 that rearranges secondary structures to fit the variance of RBMs. Furthermore, the REV7–RAN structure suggested that RAN bound to REV7 might be more accessible in GTP-bound RAN than in GDP-bound RAN. We then demonstrate that REV7 preferentially binds RanGTP instead of RanGDP using in vitro pulldown assays, in vivo coimmunoprecipitation (co-IP), and surface plasmon resonance (SPR) experiments. Our results imply that, under careful control of the GTP/GDP cycle, RAN may serve as a molecular switch by interacting with REV7 to regulate APC/C–CDH1 activity.

Results

Overall structure of the REV7–RAN complex

A previous report showed that the C-terminal moiety (residues 157–216) of RAN is responsible for the interaction with REV7 in vitro. Sequence alignment of RAN with other reported REV7-binding motifs suggested that RAN (174–185) might be the minimal RAN (Fig. 1A). To test whether RAN (174–185) is sufficient to bind REV7 in vitro, we prepared recombinant GST-tagged RAN (174–185) to pull down purified, MBP-tagged, full-length REV7. As shown in Fig. 1B, GST-RAN (174–185) could pull down MBP-REV7, whereas the GST tag alone had no binding to MBP-REV7 (Fig. 1B), demonstrating that RAN (174–185) acts as RAN(RBM).

We successfully crystallized the REV7–3–1 complex by fusion protein strategy in a previous report (11). Similarly, here we designed a fusion protein with a 10×(Gly-Ser) linker (10GS) sequence between full-length REV7 and RAN (169–185). To improve crystallization, REV7 Arg-124 was mutated to Ala, as reported previously (4), but the fusion protein was not successfully crystallized. We next engineered the sequence of a short REV3(RBM) α-helix (residues 1887–1894) after the REV7–RAN(RBM) sequence to generate the fusion protein REV7.RAN3 (Fig. 1C). We proposed that this additional REV3 α-helix could further enhance complex formation. The crystal structure of REV7.RAN3 was subsequently determined at 2.0 Å resolution by molecular replacement using the REV7–REV3 structure (PDB code 3ABD) as the search model (X-ray statistics are listed in Table 1). The crystals belonged to the P3_2_1 space group, containing one molecule in the asymmetric unit (Fig. 1D). The final model includes most residues of REV7 and residues 175–185 of RAN(RBM) (Fig. 1E), whereas the 10GS segment was untraced in the electron density map because of its flexibility. Although it facilitated the crystallization, the short REV3 α-helix was also untraced, possibly because of the lack of crystal packing contact or direct binding and exposure to the solvent channels in the crystal lattice.

The REV7–RAN(RBM) complex structure presents a closed conformation of REV7 bound with the RAN(RBM) peptide that is threaded through REV7, revealing the typical safety belt architecture seen previously in the REV7–REV3 complex structure (4, 41). The complex mainly contains two sides. One side has three REV7 α-helices (αA, αB, and αC) and a small antiparallel β-sheet comprising REV7 β2 and β3 strands, whereas the other side is an antiparallel β-sheet comprising seven strands (β4–β8 of REV7 and βRAN). The C-terminal region (residues 153–211) following β6 of REV7 is stabilized by wrapping around the entire surface of the βRAN strand (Fig. 1D).

Interaction details between REV7 and RAN(RBM)

The total buried surface area between the RAN(RBM) peptide and REV7 is about 980 Å², mainly comprising two regions, the β-sheet region and the proline core region, as shown in Fig. 2A. In the β-sheet region, RAN residues Val-177, Ala-178, and Met-179 form one β-strand (referred to βRAN), interacting with REV7’s β6 and β7 strands (residues Val-150, Ile-172, and Ala-174) by backbone hydrogen bonds, assembling into an antiparallel β-sheet. Ahead of the β-sheet, the side chain of Glu-175RAN forms one hydrogen bond with the side chain of His-151REV7, whereas Leu-175RAN and Ala-175 REV7 by backbone hydrogen bonds, assembling into an antiparallel β-sheet. The bulky side chain of Phe-176RAN wedges into the hydrophobic pocket lined up by residues in the loop connecting the REV7 β6 and β7 strands (Fig. 2, A and B). In the proline core region, Pro-180RAN stacks tightly into the hydrophobic pocket built up by Tyr-63, Phe-169, Pro-170, and Trp-171 of REV7 (Fig. 2, A and C), in which the backbone of A181RAN forms one hydrogen bond with the backbone of Pro-170REV7, and the backbone of Leu-182RAN forms another hydrogen bond with the hydroxy group in the side chain of Tyr-63REV7. Both Pro-184 and Pro-185 of...
RAN are accommodated in another large hydrophobic pocket formed by Tyr-37, His-57, Leu-60, Tyr-63, and Phe-146 of REV7 (Fig. 2A and C), in which the backbone carbonyl oxygen of Pro-185RAN forms a hydrogen bond with the hydroxy group in the side chain of Tyr-37REV7.

Mutation analysis of the REV7–RAN interaction

To confirm the binding surface of REV7 identified by the structural analysis, we purified six alanine mutants of MBP-tagged, full-length REV7 and tested their binding to the GST-RANRBM using pulldown experiments. All six mutants revealed remarkably reduced affinity between REV7 and RANRBM (Fig. 2D). Previous reports have shown that RBM can be denoted as "XX/H9021/H9021XPP," in which /H9021 is an aliphatic residue, X is any amino acid residue, and P and p are proline; proline at the 10th position is less critical than proline at the 6th and 11th positions. To further confirm the importance of the consensus residues in the RANRBM, we introduced proline-to-alanine substitutions (P180A, P184A, and P185A) into His-tagged, full-length RAN and tested their binding by MBP-Rev7 pulldown experiments (Fig. 2E). Mutations P180A and P184A caused a significant reduction in binding, whereas P185A essentially abolished REV7 binding. In addition to proline-to-alanine substitutions, we also generated a V177E mutation of full-length RAN to investigate the significance of the conserved aliphatic residues of RAN.
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Figure 2. Interaction details of the REV7–RAN complex. A, structural details of the interactions between REV7 (light blue and green) and RANRBM (magenta). The key residues are shown as sticks and labeled. Water molecules are shown as red spheres. Electrostatic interactions are shown as yellow dots. B and C, Phe-176 and Pro-180 (magenta sticks) of RANRBM bind into the hydrophobic pockets of REV7 (electrostatic representation). D, GST pulldown of MBP-tagged REV7 variants by GST-RANRBM. Top, Coomassie Blue stain of 30% input MBP-REV7 variants (arrow). Bottom, Coomassie Blue stain of the pulldown results (arrows). E, MBP pulldown of His-tagged, full-length RAN variants by MBP-REV7. Top, Coomassie Blue stain of input His-RAN variants (arrow). Bottom, Coomassie Blue stain of the pulldown results (arrows).

in the RANRBM. As expected, substitution of valine with glutamate resulted in a significant reduction. Together, these mutagenesis results are consistent with our structural analysis.

Overall structure of the REV7–IpaB complex

The IpaB fragment (residues 61–70) involved in REV7 binding has been defined previously by a yeast two-hybrid assay (40). Following the successful design of the REV7.RAN3 fusion protein, we generated a REV7.IpaB3 fusion protein (Fig. 3A); however, it failed to be crystallized. To improve the crystallization behavior, we rigidly fused a modified MBP tag with surface entropy reduction (43, 44). Details of the interaction between REV7 and IpaB

The total interface area between IpaB and REV7 is about 985 Å², as summarized in Fig. 3D. IpaB residues Ile-62, Leu-63, and Leu-64 form one β-strand (βIpaB) to assemble into an antiparallel β-sheet with REV7’s β6 and β7 strands (residues Val-150, Ile-172, and Ala-174). The side chain of Leu-63IpaB stacks into the hydrophobic pocket lined up by residues in the loop connecting the REV7 β6 and β7 strands. Ahead of the β-sheet region, Ser-60IpaB and Ala-156REV7 form one hydrogen bond. Notably, Asn-61IpaB forms three hydrogen bonds with Thr-152REV7. Following the β-sheet region, Pro-65IpaB binds into the hydrophobic pocket built up by Tyr-63, Phe-169, Pro-170, and Trp-171 of REV7, whereas Ala-79 and Pro-70 of IpaB are accommodated in another hydrophobic pocket formed by Tyr-37, His-57, Leu-60, Tyr-63, and Phe-146 of REV7. The backbones of Glu-66 and Leu-67 of IpaB form one hydrogen bond with the backbones of Pro-170REV7 and Tyr-63REV7, respectively, whereas the backbone carbonyl oxygen of Pro-70IpaB forms a hydrogen bond with the side chain of Tyr-37REV7.

REV7 employs an adaptor region to fit the variance of RBMs

The interactions of REV7 with diverse RBMs result in highly similar and well-defined conformations. However, superposition of the structures of REV7–RAN, REV7–IpaB, REV7–REV3 (PDB code 6BC8), and REV7–CAMP (PDB code 5XPT) revealed a REV7 region (156–173, named the adaptor region) whose secondary structures are notably rearranged upon binding...
diverse RBMs (Fig. 4A). This adaptor region formed a short α-helix (161–164) and one short β-strand (170–173) in the REV7–RAN complex, had a more extended α-helix (156–164) and one short β-strand (170–173) in the REV7–IpaB complex, formed two β-strands (164–167 and 169–173) in the REV7–REV3RBM2 structure, and only had one short β-strand (170–173) in the REV7–CAMP complex (Fig. 4B), implying that the adaptor region of REV7 can reshape itself according to the variance of RBMs.

Close inspection of the structural details indicates that residues in the adaptor region apply different secondary structures to form the hydrophobic pocket to fit the various steric hindrances of the side chains of residues in RBMs. In more detail, the bulky side chain of Phe-176RAN is flipped into the hydrophobic pocket lined up by residues Val-150, Met-160, Lys-162, Ile-163, and Leu-173 of REV7 (Fig. 4C, left panel). The side chain of Leu-63IpaB stacks into the hydrophobic pocket formed by residues Ile-74, Val-150, Ala-156, Met-160, Ile-163, and Trp-171 of REV7 (Fig. 4C, right panel). These observations imply that the adaptor region of REV7 is inherently versatile; that is, correlated with the residues locating at the second and fourth position of the consensus sequence XXΦΦΦXXΦXXpP.

GTP- and GDP-bound RAN obtain different binding affinities with REV7

Previous structural studies established that RanGDP presents a closed conformation, whereas RanGTP shows transfor-
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**Fig. 5**

**A** REV7-RAN, **B** REV7-IpaB, **C** REV7-REV3 (PDB 6BC8), **D** REV7-CAMP (PDB 5XPT). The C-terminal moiety (residues 156–210) of RAN (29, 45). We noticed that RAN RBM locates within the hinge loop of the C-terminal moiety, implying that the closed conformation of RanGDP may render its C-terminal moiety inaccessible for REV7 binding, so it is highly possible that RanGTP has a higher affinity than RanGDP in binding REV7.

To test this hypothesis, we introduced mutations T24N and L43E into full-length, His-tagged RAN to serve as RanGDP and RanGTP mimetics, respectively (26, 46–48), and tested their binding by MBP-REV7 pulldown experiments (Fig. 5A). RanGTP mimicking RAN (L43E) revealed slightly enhanced REV7 binding compared with WT RAN, whereas RanGDP mimicking RAN (T24N) resulted in a significant reduction in REV7 binding compared with WT RAN or RAN (L43E), demonstrating that RanGTP has a stronger REV7-binding ability than RanGDP. Validation of these results in vivo using co-IP performed in HEK293 cells showed clear binding of WT HA-RAN to yellow fluorescent protein–REV7, which was reduced in the HA-RAN (T24N) mutant (Fig. 5B). Furthermore, to show that RAN can regulate binding of CDH1 to APC/C, we cotransfected HEK293 cells with myc-CDH1 or double-transfected them with myc-CDH1 and HA-RAN and performed co-IP against CDC27. Only after double transfection did we observe an increase in CDH1 binding to Cdc27, suggesting that HA-RAN binds endogenous REV7, releasing myc-CDH1 to bind to Cdc27 (Fig. 5C).

To further confirm that RAN bound with GTP favors the interaction with REV7, we used SPR experiments to determine the affinities of MBP-REV7 with different His-RAN proteins (WT, T24N, or L43E). Purified MBP-REV7 or MBP-tag alone was immobilized on a CM5 chip. Two-fold increasing concentrations of purified His-RAN proteins were injected and examined. Consistent with the pulldown and co-IP experiments above, SPR results also showed that REV7 binds RAN (L43E) with the highest affinity ($K_d = 0.90 \mu M$), whereas REV7 binds WT RAN ($K_d = 1.85 \mu M$) and RAN (T24N) ($K_d = 2.80 \mu M$) with lower affinities (Fig. 5D). In summary, these results support the statement that REV7 has a marked binding preference of RanGTP to RanGDP and that RAN can regulate the amount of free MAD2L2 to enable CDH1 binding to APC/C.

**Discussion**

Different aspects of REV7’s regulatory role in mitosis together with novel binding proteins are emerging, emphasizing the complexity of its function. The GTPase RAN is known to be a REV7-interacting protein, and their interaction is important for assembly of the mitotic spindle. Based on our findings, we propose a model where REV7 preferentially binds to the RAN-GTP conformation (Fig. 6). This model emphasizes that the switch of RAN-GDP to RAN-GTP exposes its C-terminal moiety to enable REV7 binding (Fig. 6A). With the presence of RAN-GTP, REV7 undergoes structural rearrangement of the safety belt upon RAN RBM binding to form the stable REV7–RAN–GTP complex (Fig. 6B). We speculate that the GTP/GDP cycle of RAN may serve as a molecular switch for controlling the levels of REV7 and regulate REV7-related mechanisms; the APC/C activity and the interaction of other partners with REV7, most of which have biological functions, are still unclear. The RAN RBM is structurally similar to the REV3 RBM and CAMP RBM, and all contain the identified REV7-binding motif $\chi_1\Phi\chi_2\chi_3\chi_4\chi_5p$. Free REV7 can adopt an open-form conformation; however, it is not clear how stable free REV7 is and what the moiety of the free protein is in the cell. The mechanism that regulates and determines the binding pattern of REV7 to its TLS nonhomologous end joining or mitotic partners has yet to be revealed. Dissecting different binding residues and subtle conformational changes will be crucial for our understanding of the complexity of REV7 function and facilitate future development of targeted therapies against REV7 in specific pathways.
Experimental procedures

Protein expression and purification

REV7.RAN3 was designed to contain the full-length human REV7 (with the mutation R124A), the 10\(^\text{Gly-Ser}\) linker, RAN (169–185), and Rev3 (1887–1894). The sequence encoding REV7.RAN3 was cloned into the pQLinkH plasmid with an N-terminal His\(_6\) tag. The protein was expressed by *Escherichia coli* XL10gold cells. Bacteria were grown at 37 °C to 0.8 \(A_600\) and induced by 0.2 mM isopropyl 1-thio-D-galactopyranoside (IPTG) at 23 °C overnight. The protein was purified by nickel affinity chromatography, and the His\(_6\) tag was removed by incubation with tobacco etch virus protease during dialysis to buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM DTT) overnight at 4 °C. The protein was further purified by anion exchange (Resource Q) and size exclusion chromatography (Superdex 200 (16/60) column) equilibrated with buffer (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 1 mM TCEP). Proteins were concentrated to about 10 mg/ml and stored at −80 °C.

REV7.IpaB3 was designed to contain the human REV7 (13–211 with the mutation R124A), the 10\(^\text{Gly-Ser}\) linker, IpaB (50–76), and Rev3 (1887–1894). The sequence encoding REV7.IpaB3 was cloned into the pMALX plasmid with an N-terminal modified MBP tag (42). The protein was expressed by *Escherichia coli* XL10gold cells. Bacteria were grown at 37 °C to 0.8 \(A_600\) and induced by 0.2 mM isopropyl 1-thio-D-galactopyranoside (IPTG) at 23 °C overnight. The protein was purified by nickel affinity chromatography, and the His\(_6\) tag was removed by incubation with tobacco etch virus protease during dialysis to buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM DTT) overnight at 4 °C. The protein was further purified by anion exchange (Resource Q) and size exclusion chromatography (Superdex 200 (16/60) column) equilibrated with buffer (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 1 mM TCEP). Proteins were concentrated to about 30 mg/ml and stored at −80 °C.

Figure 5. RanGTP shows higher affinity for REV7 than RanGDP. A, MBP pulldown of His-tagged, full-length RAN variants by MBP-tagged REV7. Mutant RAN (T24N) is a mimetic of RanGDP, and mutant RAN (L43E) is a mimetic of RanGTP. Top, Western blot (WB) of input His-RAN variants using anti-His antibodies. Bottom, Western blot of the pulldown results using anti-His and anti-MBP antibodies. B, HEK293 cells were double-transfected with yellow fluorescent protein–REV7 and HA-tagged RAN WT or HA-tagged RanGDP (T24N), and *in vivo* co-IP against REV7 was performed. C, HEK293 cells were transfected with myc-CDH1 or double-transfected with myc-CDH1 and HA-tagged RAN WT. *In vivo* co-IP against Cdc27 was performed. D, SPR analyses of REV7–RAN interactions. Purified MBP-REV7 was immobilized on a CM5 chip. The affinities were evaluated over a concentration range from 0.625 \(\mu\)M to 20 \(\mu\)M of His-RAN variants in 2-fold increments. The concentration of 0.625 \(\mu\)M was repeated as an internal control. The calculated \(K_d\) values are indicated. RU, resonance units.

Figure 6. RanGTP exposes its C-terminal moiety to bind REV7. A, structure comparison of RanGDP and RanGTP. The cartoon models of RanGDP (PDB code 3GJ0) and RanGTP (PDB code 1K5D) are colored wheat and orange (the C-terminal moiety), and their REV7-binding motifs are colored magenta and labeled. B, model of the conformation changes in REV7–RAN binding. The conformation change from RanGDP to RanGTP exposes its C-terminal moiety. Free REV7 adopts an open-form conformation. Upon binding RanGTP, the C-terminal moiety of REV7 also undergoes structural rearrangement to form safety belt architecture in the REV7–RanGTP complex.
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**Crystallization and structure determination**

REV7.RAN$_3$ was crystallized by the hanging drop vapor diffusion method to equilibrate 1.0 µl of REV7.RAN$_3$ solution (about 7.5 mg/ml) with 1.0 µl of reservoir solution (100 mM HEPES (pH 7.0), 1.1 M sodium malonate, and 0.5% Jef-famine ED-2001). Crystals appeared after 2 days of incubation at 16 °C and were improved further by the microseeding method. The crystals were harvested into the cryoprotectant solution containing Paratone-N mixed with paraffin oil at a 1:2 volume ratio for a few seconds before being flash-frozen by liquid nitrogen. X-ray diffraction datasets were collected on Beamline BL18U at the Shanghai Synchrotron Radiation Facility. The data were processed and scaled using the HKL3000 software package. The structure was then determined by molecular replacement in PHENIX Phaser (49, 50) using the REV7–REV3 complex structure (PDB code 3ABD) as the search model. Iterative rounds of model building and refinement were performed in COOT (51) and PHENIX. Refine (52). Structure factors and final coordinates were deposited (PDB code 6NIF). Data collection and refinement statistics are shown in Table 1.

REV7.IpaB$_3$ was crystallized by the hanging drop vapor diffusion method to equilibrate 1.5 µl of REV7.IpaB$_3$ solution (about 20 mg/ml) with 1.5 µl of reservoir solution (100 mM BisTris (pH 5.6) and 25% PEG3350). The crystals were harvested into cryoprotectant solution containing 30% glycerol before being flash-frozen by liquid nitrogen. X-ray diffraction data were collected on the 24-ID-C beamline at the Advanced Photon Source. The data were autoprocessed by the NECAT RAPD online server. The structure was then determined by molecular replacement in PHENIX Phaser (49, 50) using the MBP (PDB code 3VDB) and REV7–REV3 structures (PDB code 3ABD) as the search models. Iterative rounds of model building and refinement were performed in COOT (51) and PHENIX. Refine (52). Structure factors and final coordinates were deposited (PDB code 6KEA). Data collection and refinement statistics are shown in Table 1.

**Pulldown assays**

For GST–RAN$_{RBM}$ pulldown, bacterial cells expressing GST–RAN$_{RBM}$ were lysed by sonication in buffer W (50 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 1 mM DTT). After centrifugation, a 1-ml aliquot of supernatant was incubated with 20 µl of GSH-Sepharose resin (GE Healthcare) for 30 min at 4 °C. The beads were then washed four times with 1 ml of buffer W. WT or mutant MBP–REV7 was purified by amylose resin (New England Biolabs) and eluted with buffer W supplemented with 10 mM maltose. Then a 500-µl aliquot of 1 mg/ml WT or mutant MBP–REV7 was added to the GST–RAN$_{RBM}$–bound beads and incubated for 1 h at 4 °C. The beads were collected by centrifugation (5 min, 500 × g, 4 °C) and washed five times in 1 ml of buffer W with 0.05% (v/v) Tween 20. Bound proteins were eluted with 40 µl of SDS-PAGE loading buffer and analyzed by Coomassie Blue–stained SDS-PAGE.

For MBP–REV7 pulldown, 1 ml of supernatant was mixed with 20 µl of amylose resin (New England Biolabs) in buffer W. The beads were then washed four times with the same buffer. His-tagged WT or mutant full-length RAN was purified by nickel–nitriilotriacetic acid beads (Roche) and incubated with MBP–REV7–bound amylose resin for 1 h at 4 °C. Bound proteins were eluted with 40 µl of SDS-PAGE loading buffer and analyzed by Coomassie Blue–stained SDS-PAGE or Western blotting.

**Analytical SEC**

Analytical size-exclusion chromatography was performed using the AKTA FPLC system (GE Healthcare) on a Superdex200 10/300 gel filtration column (GE Healthcare). His-tagged REV7, REV7–10GS, or REV7–10GS–REV7–IpaB$_3$ proteins were purified by nickel affinity chromatography. The eluted proteins were concentrated and injected into the gel filtration column pre-equilibrated with buffer (20 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 1 mM TCEP).

**SFR experiments**

Interactions of MBP–REV7 with His-tagged RAN, RAN (T24N), and RAN (L43E) were measured on a BIACore 3000 instrument (BIACore AB, Uppsala, Sweden). MBP–REV7 was immobilized on the carboxymethylated dextran surface–modified chip (CM5 chip) according to the amine-coupling protocol of the BIACore manual. The running buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, and 0.005% (v/v) Tween 20) was filtered through Millipore Film (pore size, 0.22 mm) and degassed before use. The binding affinities were evaluated over a concentration of RAN from 0.625 µM to 20 µM in 2-fold increments at 25 °C. For three binding assays, the concentration of 0.625 µM was repeated as an internal control. All of the data collected were analyzed using BIAnalysis software version 4.1 using a 1:1 Langmuir binding model.

**Cell culture and transfections**

For experiments using the human HEK293 cell line, cells were grown in DMEM supplemented with 2 mM l-glutamine, penicillin/streptomycin and 10% fetal bovine serum (Biological Industries) and maintained at 37 °C in a humidified incubator with 5% CO². HEK293 cells were transfected with vectors containing the cloned gene of interest using Avalanche® Everyday Transfection Reagent (EZT-EVDY-1).

**Western blotting, immunoprecipitation, and antibodies**

For immunoblotting and immunoprecipitation, cells were lysed in extraction buffer (50 mM Tris–HCl (pH 8), 150 mM NaCl, 20 mM EGTA, 50 mM NaF, and 1% Triton X-100) supplemented with protease inhibitor mixture (Calbiochem, 539134). Cells were lysed on ice for 10–30 min and cleared by centrifugation at 20,000 × g for 10–30 min at 4 °C. For immunoprecipitation, clarified lysates were supplemented with REV7 (612266, BD Biosciences) or CDC27 (610454, BD Biosciences) and incubated for 1–2 h at 4 °C. Next, 30 µl of equilibrated protein G Plus agarose beads (Calbiochem IP04) was added for 1 h. Finally, the beads were washed three times in PBS and 0.1% Tween 20 and boiled in Laemmli buffer for 5 min.
Structural insights into REV7-RAN and REV7-IpaB complexes

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