Structure of the RAD9-RAD1-HUS1 checkpoint clamp bound to RHINO sheds light on the other side of the DNA clamp

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The atomic coordinates and structure factors (code 6J8Y)‡ have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Results and discussion

The RHINO peptide is bound to the edge and around the back of 9-1-1

RHINO is known to form a stoichiometric complex with 9-1-1 through interactions with RAD9 lacking the C-terminal tail and RAD1 (16). Residues S5WVSPDF of RHINO are crucial for this interaction with 9-1-1 and activation of the checkpoint (15). Thus, to elucidate details of the interaction between 9-1-1 and its binding proteins, we determined the X-ray crystal structure of the 9-1-1 core ring bound to a RHINO peptide.
(S5KPIDHSTSWVSPDFDTA64) at 2.4 Å resolution (Fig. 1A and Table 1). Electron density map shows that residues 55–64 are well-ordered, whereas the N-terminal region (residues 45–54) is disordered (Fig. 1B). In the structure, the RHINO peptide adopts an extended conformation and is bound to the RAD1 subunit. Unexpectedly, the peptide is bound to the edge and around the back of the 9-1-1 ring (Fig. 1A, left). This interaction is distinct from that of known clamp-peptide complexes, such as PCNA in complex with a p21 peptide, where the p21 peptide including the PIP-box is bound to a binding pocket underneath the IDC-loop on the front of PCNA (Fig. 1C).

**The RHINO peptide specifically interacts with the RAD1 subunit**

The RHINO peptide is accommodated in the pocket on the edge and around the back of the RAD1 subunit of 9-1-1, and the chemical properties of the bound peptide are complementary to the charge distribution of the binding pocket (Fig. 2A). The hydrophobic side chains of Trp56, Val57, Pro59, and Phe61 of RHINO interact significantly with the pocket of RAD1 through van der Waals (vdW) contacts, as follows (Fig. 2B). Trp56 of RHINO contacts with the side chain of Phe64 of RAD1 by a face-to-edge interaction. Val57, Pro59, and Phe61 of RHINO contact with the hydrophobic side chains of Phe64, Phe266, and Met256 of RAD1, respectively, by CH-π interactions. The methyl group of Thr63 of RHINO is also accommodated into the hydrophobic pocket. In addition to these vdW interactions, the acidic side chain of Asp60 of RHINO forms ion pairs with the basic side chains of Lys155 and Arg244 of RAD1 (Fig. 2B), which contribute to the basic patch on the surface (Fig. 2A).

**Table 1**

| Parameters | 9-1-1–RHINO |
|------------|-------------|
| **Data collection** | |
| Space group | P21,2,21 |
| Cell dimensions | |
| a, b, c (Å) | 52.9, 136.1, 154.0 |
| Resolution (Å) | 19.80–2.40 (2.48–2.40) |
| Rmerge | 0.062 (0.733) |
| Rmeas | 0.067 (0.796) |
| I/|I| | 21.1 (2.3) |
| CC1/2 | 99.9 (77.9) |
| Completeness (%) | 99.6 (99.5) |
| No. of reflections | 44,421 (6,970) |
| Redundancy | 6.7 (6.7) |

| **Refinement** | |
| Resolution (Å) | 19.8–2.40 |
| No. of reflections | 44,415 |
| Rwork | 0.2184 |
| Rfree | 0.2581 |
| No. of atoms | |
| 9-1-1 | 6,120 |
| RHINO peptide | 79 |
| Water | 68 |
| B-factors | |
| 9-1-1 | 63.5 |
| RHINO peptide | 74.0 |
| Water | 53.9 |
| Root mean square deviations | |
| Bond lengths (Å) | 0.003 |
| Bond angles (degrees) | 0.70 |
| PDB entry | 6J8Y |

**Figure 1.** A, overall structure of 9-1-1 bound to the RHINO peptide. RAD9, RAD1, and HUS1 subunits are shown as green, cyan, and magenta cartoons, respectively. The IDC-loop of each subunit is shown as a darker-colored thick tube. The RHINO peptide is shown as a yellow stick. Left, back view of 9-1-1 bound to the RHINO peptide. Center, lateral view of 9-1-1 bound to the RHINO peptide. For clarification, RAD9 and HUS1 are shown as semitransparent models. The C terminus and IDC-loop of RAD1 are indicated by arrows. Right, front view (C-side) of 9-1-1 bound to the RHINO peptide. The orientation PCNA in each view corresponds to that of 9-1-1 in A.

B, the electron density map of the RHINO peptide bound to RAD1. The weighted 2Fo–F1 map of the peptide contoured at 1σ is shown as a purple cage. RHINO, RAD9, and RAD1 are colored as shown in A. Residues of the RHINO peptide are labeled. C, overall structure of PCNA bound to the p21 peptide (PDB entry 1AXC) as a representative structure of the PCNA-partner complex. Subunits of the homotrimer are shown as light green, pale blue, and pink cartoons. The IDC-loop of each subunit is shown as darker-colored thick tube. The p21 peptide is shown as an orange tube. Left, lateral view of PCNA bound to the p21 peptide. For clarification, far subunits (light green and pink) are shown as transparent models. The C terminus and IDC-loop of PCNA are indicated by arrows. Right, front view (C-side) of PCNA bound to the p21 peptide. The orientation PCNA in each view corresponds to that of 9-1-1 in A.
Furthermore, hydrogen bonds between the peptide bond of Pro$^{59}$-Asp$^{60}$ of RHINO and the side chain of Gln$^{254}$ of RAD1 are also observed. The bidentate hydrogen bond by Gln$^{254}$ retains the conformation of the peptide bond, thereby probably stabilizing the conformation for binding. Furthermore, the residues of RHINO involved in interaction with RAD1 are highly conserved (Fig. 2C).

For functional validation of the present structure, we performed interaction analysis using a GST pulldown assay with triple mutations (m1 (K155A/R244A/Q254A) or m2 (F64A/M256A/F266A)) in the RAD1 subunit (Fig. 3). First, we confirmed binding of RHINO(45–64) to 9-1-1 (Fig. 3, lane 7). The triple mutations in Lys$^{155}$, Arg$^{244}$, and Gln$^{254}$, which are engaged in electrostatic interactions with the RHINO peptide (Fig. 2B), reduced the binding (Fig. 3, lane 8). Furthermore, the triple mutations in Phe$^{64}$, Met$^{256}$, and Phe$^{266}$, which are involved in vdW contacts with the RHINO peptide (Fig. 2B), largely reduced the binding (Fig. 3, lane 9). These results are in good agreement with the crystal structure, and thus we conclude that the RHINO peptide is specifically bound to the pocket of RAD1. In addition, the pulldown assay might indicate that vdW contacts are more crucial for the binding.

In contrast to PCNA, 9-1-1 is a heterotrimer, and thus the molecular surface of each subunit possesses different physico-chemical properties, whereas RAD9, HUS1, and PCNA also possess large concaves that seem to be available for a binding site on their backs and edges. Thus, by structural superimposition of RAD9, HUS1, and PCNA with RAD1 bound to the
ACCELERATED COMMUNICATION: The other side of 9-1-1 DNA clamp

DNA clamps are loaded on dsDNA by specific clamp loaders, which are pentameric protein complexes classified into the AAA+-ATPase family (18). Eukaryotes have four clamp loaders: RFC, CTF18-RFC, ELG1-RFC, and RAD17-RFC (19). RFC, the primary clamp loader for PCNA, consists of one large and four small subunits: RFC1 and RFC2-5, respectively. The others consist of the RFC1 paralog as a large subunit and CTF18, ELG1, and RAD17, associated with universal small subunits, RFC2-5. The crystal structure of yeast homolog of the RFC-PCNA complex has revealed that RFC also binds to the front of PCNA for ring opening and loading of PCNA on dsDNA (20). In contrast to PCNA, 9-1-1 is loaded on dsDNA by RAD17-RFC (21). Based on sequence similarity and crystal structures, plausible models of interaction between RFC17-RFC and 9-1-1 have been proposed (10, 12, 13). These models suggest that RAD17-RFC also interacts with the front of 9-1-1. A preliminary result also implies that FEN1 nuclease might be bound to the front of RAD1 (12). Here, our structure reveals that the RHINO specifically interacts with the edge and back of 9-1-1 for activation of the checkpoint. Therefore, 9-1-1 could be a functionally double-faced DNA clamp and thereby might enable multiple protein-protein interactions in signaling of the DNA damage checkpoint. Although RHINO is unlikely to interact with the edge and back of PCNA (Fig. 4), we cannot exclude the possibility that PCNA utilizes its edge or back for protein-protein interactions. Further studies are needed to discuss that possibility.

Conclusion

In this study, we have determined the crystal structure of 9-1-1 in complex with the RHINO peptide. Most surprisingly, the structure reveals that 9-1-1 utilizes its edge and back to interact with RHINO. Our unexpected finding first sheds light on the other side of 9-1-1, thereby indicating that 9-1-1 is a functionally double-faced DNA clamp and fundamentally changing our view of DNA clamps. The structure also reveals detailed interactions between 9-1-1 and RHINO and thus could provide a new clue for developing chemical compounds or artificial peptides to down-regulate the ATR-CHK1 checkpoint pathway for cancer therapy.

Experimental procedures

Protein production

The gene encoding human truncated RAD9 (residues 1–270), RAD9AC, was inserted into the pCOLA vector. The whole genes encoding HUS1 (residues 1–280) and RAD1 (residues 1–282) were inserted into the pETDuet vector, which incorporated a His tag at the N terminus of HUS1. Escherichia coli BL21(DE3) harboring the two vectors was cultured in lysogeny broth medium at 37 °C to an optical density of about 0.8 at 660 nm, 0.2 mM isopropyl 1-thio-β-D-galactopyranoside was added, and the cells were then cultured at 25 °C overnight.

The cells were harvested by centrifugation, washed with buffer I (20 mM HEPES-NaOH, pH 7.4, and 500 mM NaCl), and resuspended in buffer II (50 mM HEPES–NaOH, pH 6.8, and 250 mM NaCl). After sonication, the cell lysate was clarified by centrifugation for 1 h at 4 °C (48,300 × g). Subsequent purification was
carried out at 4 °C. The supernatant was applied to a HiTrap Heparin column (GE Healthcare), and bound protein was eluted with a linear gradient of 250 mM to 1M NaCl. The eluent was diluted with buffer III (50 mM Tris-HCl, pH 8.5, and 200 mM NaCl) and applied to an anion-exchange column, HiTrap Q column (GE Healthcare). The bound protein was eluted with a linear gradient of 200 mM to 1M NaCl. The eluted protein was passed through a size-exclusion column, HiLoad 16/600 Superdex 200 (GE Healthcare), equilibrated with buffer IV (20 mM HEPES-NaOH, pH 7.4, and 100 mM NaCl), and then concentrated to 15–20 mg/ml using a Vivaspin concentrator (Sarto-tech). The purity of the RAD9/H9004 C-RAD1-HUS1 complex (9/H9004 C-1-1) was confirmed by SDS-PAGE with Coomassie Brilliant Blue staining. In the present study, RAD9/H9004 C and 9/H9004 C-1-1 are simply termed RAD9 and 9-1-1, respectively, unless otherwise noted. For interaction analysis, site-directed mutations in RAD1 (m1 (K155A/R244A/Q254A) or m2 (F64A/M256A/F266A)) were introduced by a PCR-based mutagenesis. Recombinant 9/H9004 C-1-1 with RAD1 mutations was produced and purified by a procedure similar to that of the WT. All purified proteins were frozen in liquid N2 and stored at −80 °C until use.

**Crystallization and structure determination**

The RHINO peptide (E5SKPIDHSTTSVSPDFDTA) was commercially synthesized (TORAY Research Center, Inc.) and dissolved in buffer IV. A 10-fold molar excess of peptide was incubated with 0.13 mM 9-1-1 and 8.8 mM DTT. Crystallization was performed by the vapor-diffusion method. Rod crystals were obtained with a reservoir solution of 0.1 M Bistris propane, pH 7.5, 0.1 M sodium citrate, and 16% PEG 3350 at 20 °C. Crystals were transferred to a buffer containing the reservoir solution and 25% sucrose. X-ray diffraction data were collected at Photon Factory beamline BL-17A (Tsukuba, Japan) with an Eiger X16M single-photon-counting detector (DECTRIS). Diffraction data were processed with the program XDS (22). The crystal structure of 9-1-1 bound to the RHINO peptide was determined by molecular replacement method using the program PHASER (23). Binding of the RHINO peptide to the RAD1 subunit was confirmed by difference Fourier map, and the structure of the peptide was built by the program Coot (24). The structure was manually improved and refined by the program PHENIX (25). The final electron density of the RHINO peptide is shown in Fig. 1B. Data collection and refinement statistics are given in Table 1. Atomic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (entry 6J8Y).

**GST pulldown assay**

GST or GST-fused RHINO (residues 45–64) termed GST-RHINO(45–64) was expressed by E. coli BL21(DE3) by conventional induction with isopropyl 1-thio-β-D-galactopyranoside. The cell lysates expressing GST or GST-RHINO(45–64) were incubated with GSH-Sepharose 4B (GE Healthcare) beads for 2 h at 4 °C. The beads were washed four times with buffer H (25 mM HEPES-NaOH (pH 7.8), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml leupeptin). The beads immobilized with ~50 pmol of GST or GST-RHINO(45–64) were incubated with 100 pmol of 9AC-1-1 (WT) or its mutants (m1 and m2) for 2 h at 4 °C in 25 µl of buffer H, followed by five washes with the same buffer. The bound proteins were eluted by boiling in SDS sample buffer (50 mM Tris-HCl (pH 6.8), 0.1 mM DTT, 2% SDS, 0.05%
bromphenol blue, and 10% glycerol), separated by 12.5% SDS-PAGE, and analyzed by immunoblotting using the indicated antibodies. The band intensities were quantified by ImageJ software (National Institutes of Health).

**Antibodies**

The rabbit anti-HUS1 and anti-RAD1 antibodies were gifts from Dr. Katsunori Sugimoto (State University of New Jersey). The rabbit anti-RAD9, mouse anti-GST, horseradish peroxidase–conjugated anti-rabbit IgG, and Alexa 647–conjugated antirat IgG antibodies were purchased from LifeSpan Biosciences, Santa Cruz Biotechnology, Bio-Rad, and Jackson Immuno-Research Laboratories, respectively.

**Figure preparation**

Figures of protein structures were prepared with the program PyMOL (Schrödinger, LLC). All figures were modified with the program Illustrator (Adobe Systems).

**Author contributions**—K. H., N. I., R. T., E. O., H. S., and A. H. investigation; K. H., E. O., and H. H. validation; H. H. conceptualization; K. H. and E. O. curation; K. H. and E. O. formal analysis; K. H. figure administration; K. H., E. O., Y. I., A. H., and H. H. writing-review and editing.

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