Structures of the Human Rad17-Replication Factor C and Checkpoint Rad 9-1-1 Complexes Visualized by Glycerol Spray/Low Voltage Microscopy*

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Human checkpoint Rad proteins are thought to function as damage sensors in the DNA damage checkpoint response pathway. The checkpoint proteins hRad9, hHus1, and hRad1 have limited homology to the replication processivity factor proliferating cell nuclear antigen (PCNA), and hRad17 has homology to replication factor C (RFC). Such observations have led to the proposal that these checkpoint Rad proteins may function similarly to their replication counterparts during checkpoint control. We purified two complexes formed by the checkpoint Rad proteins and investigated their structures using an electron microscopic preparative method in which the complexes are sprayed from a glycerol solution onto very thin carbon foils, decorated in vacuo with tungsten, and imaged at low voltage. We found that the hRad9, hHus1, and hRad1 proteins make a trimeric ring structure (checkpoint 9-1-1 complex) reminiscent of the PCNA ring. Similarly we found that hRad17 makes a heteropentameric complex with the four RFC small subunits (hRad17-RFC) with a deep groove or cleft and is similar to the RFC clamp loader. Therefore, our results demonstrate structural similarity between the checkpoint Rad complexes and the PCNA and RFC replication factors and thus provide further support for models proposing analogous functions for these complexes.

In eukaryotes, DNA damage activates a signal transduction pathway referred to as the DNA damage checkpoint, which arrests cell cycle progression to allow time for the damage to be repaired and thus ensures that an intact, fully replicated genome will be inherited. Currently an area of major interest is the mechanism by which the DNA damage is sensed. Genetic evidence indicates that Rad17, Rad9, Hus1, and Rad1 may play a role in the DNA damage-sensing step of the checkpoint response, although the mechanism remains elusive. Rad17 has homology to and makes a complex with the four RFC small subunits, and therefore, it has been proposed that this RFC-like complex functions similarly to RFC yet is specialized for damage response (1–6). Similarly, although Rad9, Hus1, and Rad1 exhibit limited sequence homology to PCNA, molecular modeling analysis and interaction studies have led to the proposal that these three subunits make a PCNA-like complex with specialized functions for damage sensing (7–14). Recent reports from yeast and humans have shown that the checkpoint 9-1-1 complex associates with chromatin at treatment with DNA-damaging agents (15) and that this association is dependent on Rad17 (16–18). We previously isolated the hRad17-RFC complex and demonstrated that it has DNA-stimulated ATPase activity similar to the classical RFC (6). We also purified the checkpoint 9-1-1 complex and showed that it interacts with the hRad17-RFC complex. These findings gave credence to the notion that the checkpoint Rad proteins formed RFC/PCNA-like clamp loader/sliding clamps specialized for the checkpoint response. However, it was not known whether the checkpoint Rad proteins formed structures similar to those of RFC and PCNA. In fact, some recent results from analysis of mutant hus1, rad1, and rad9 in yeast raised questions against the PCNA-like structure of the 9-1-1 complex (19). To ascertain whether the checkpoint proteins are structurally analogous to the RFC/PCNA pair, we isolated the hRad17-RFC and checkpoint 9-1-1 complexes and analyzed them by electron microscopy (EM). Our results show that, similar to PCNA (20, 21), the checkpoint 9-1-1 complex forms a flat ring structure with a very distinct hole, whereas the hRad17-RFC complex, similar to RFC (22), is more compact but with a deep groove or cleft.

**MATERIALS AND METHODS**

Expression and Purification of the Checkpoint Complexes—Baculoviruses used for expression of FLAG-hRad17, FLAG-hRad9, hHus1, and hRad1 were described previously (6), and baculoviruses used for expression of RFC p40, Hisp-p38, p37, and p36 were the kind gift of J. Hurwitz (23). Monolayer High Five insect cells grown in Grace’s insect medium (Invitrogen) supplemented with 10% fetal bovine serum and 100 units of penicillin and streptomycin/ml were infected with a multiplicity of infection of five for each virus and then harvested after 48 h. The cells were washed with phosphate-buffered saline and lysed in 20 packed cell volumes of lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5 mM Nonidet P-40, protease inhibitors (Roche Molecular Biochemicals)) with 0.3 M NaCl for the checkpoint 9-1-1 complex and 1 M NaCl for the hRad17-RFC complex. After a 15-min incubation on ice, the cell lysate was centrifuged for 30 min at 32,000 × g. The checkpoint 9-1-1 complex supernatant was incubated with anti-FLAG agarose for 4 h at 4 °C. The resin was then washed three times with lysis buffer and then eluted with elution buffer (50 mM Tris-HCl (pH 7.5), 0.5 M Nonidet P-40, protease inhibitors (Roche Molecular Biochemicals)) with 0.3 M NaCl for the checkpoint 9-1-1 complex and 1 M NaCl for the hRad17-RFC complex.

**Electron Microscopy—**The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 mM ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed onto thin droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest Microscopy—The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 M ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed into tiny droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest Microscopy—The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 M ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed into tiny droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest Microscopy—The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 M ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed into tiny droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest Microscopy—The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 M ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed into tiny droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest Microscopy—The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 M ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed into tiny droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest Microscopy—The purified checkpoint complexes were exchanged into a buffer of 40% glycerol, 0.2 M ammonium bicarbonate, pH 7.5 by gentle centrifugation through Sephadex G-50 equilibrated in this buffer. Aliquots (10 μl) at 40 μg of protein/ml were sprayed into tiny droplets directly onto very thin, glow discharge-treated carbon foils supported by 400-mesh copper grids using an EFPA atomizer (Earnest
Fullam Inc.). The samples were placed in an oil-free cryopumped evaporator and dried for 18 h at a final vacuum of \(1 \times 10^{-8}\) torr. Without breaking the vacuum, the sample was rotary shadowcast with a thin layer of tungsten and examined immediately (to avoid hydration of the tungsten in air) in a Philips CM12 instrument at an accelerating voltage of 15–18 kV. Photographs were taken on sheet film, and examples for publication were digitized using a Nikon D-1. Panels for publication were arranged using Adobe Photoshop.

RESULTS AND DISCUSSION

**Purification of Checkpoint Rad Complexes**—We previously reported the isolation of hRad17-RFC and checkpoint 9-1-1 complexes (6). Here we have purified these complexes (Fig. 1) and determined them to be >90% pure as visualized by Coomassie Blue staining. Determination of the molecular mass of the checkpoint 9-1-1 complex by analytical centrifugation yielded a value of 110 kDa (data not shown) in good agreement with there being one copy each of the three subunits. Gel filtration analysis however suggested a nearly 2-fold higher molecular mass (were the complex spherical) indicating that the checkpoint 9-1-1 complex has a highly asymmetric shape. Similar analysis of the hRad17-RFC complex was consistent with a heteropentameric complex of hRad17 associated with the four RFC small subunits (hRad17-RFC) and a shape that is less asymmetric than the checkpoint 9-1-1 complex.

**FIG. 1.** Purification of recombinant checkpoint Rad complexes. The checkpoint Rad complexes were reconstituted in insect cells and purified by chromatography with nickel-nitrilotriacetic acid and anti-FLAG agarose as described under “Materials and Methods.” The proteins were visualized after 15% SDS-PAGE by silver staining. Lane 1, 0.1 \(\mu\)g of the checkpoint 9-1-1 complex: FLAG-hRad9, hHus1, and hRad1. As reported previously (6), hRad9 exists as multiply phosphorylated forms that are indicated by a bracket. Lane 2, 0.15 \(\mu\)g of the hRad17-RFC complex: FLAG-hRad17, p40, His-p38, p37, and p36. The minor bands below hRad17 are degradation products that make up less than 10% of the protein as determined by Coomassie Blue staining.

**FIG. 2.** Visualization of the checkpoint 9-1-1 complex by glycerol spray/low voltage transmission EM. A suspension of purified checkpoint 9-1-1 complexes in 40% glycerol was sprayed onto thin carbon foils, dried in vacuo, and rotary shadowcast with tungsten (see “Materials and Methods”). Samples were examined at 16 kV for enhanced contrast. **A**, a field of lightly shadowed checkpoint 9-1-1 particles; **B**, a gallery of individual particles showing the central hole (e.g. panels 1–4), three subunits (panels 5 and 9), and occasional C-shaped forms (panel 11). Shadowing in **B** is significantly greater than in **A** lending to the apparent larger size in addition to the greater magnification in **B**. Micrographs are shown in reverse contrast. **Bar** = 32 nm (A) and 25 nm (B).
Checkpoint 9-1-1 Ring—The EM preparative method originally described independently by Erickson and Branton and colleagues (24, 25) in which a protein sample is sprayed onto mica from a solution of 40% glycerol has the advantage of controlled drying and not exposing the protein to chemical fixatives or strong metal salts. However, for the complexes described here which are relatively small, we found that the preparation of a platinum/carbon replica from the mica provided relatively low resolution due to the size of the platinum grains and the thick carbon support. To achieve higher resolution, we sprayed the sample directly onto very thin (≤10 nm) carbon foils supported by copper grids, and following drying in an oil-free vacuum, the sample was decorated with a thin coating of tungsten. Finally, to further enhance the contrast between the complexes and background, the samples were examined at low accelerating voltage (15–18 kV) in the transmission EM in contrast to the usual 60–100 kV. Using this new approach, examination of fields of checkpoint 9-1-1 complexes revealed a large number of ring-shaped molecules (Fig. 2A), a field of hRad17-RFC particles; the arrow indicates a particle with a small hole. B, a gallery of individual particles; arrows indicate a deep cleft or groove. Micrographs are shown in reverse contrast. Bar = 35 nm (A) and 25 nm (B).

DNA polymerase I has a molecular mass (110 kDa) the same as the checkpoint 9-1-1 complex and is roughly spherical with a 6.5-nm diameter (26). Volume comparisons show that if the checkpoint 9-1-1 complex is a 10-nm ring with a 3-nm hole and 2–3-nm height that it would have a volume (hence mass) similar to that of a 6.5-nm sphere. Because the rings consist of three nonidentical subunits, simple image averaging by rotation would not be useful. Possibly in the future more complex analysis of a much larger number of molecules might yield a refined structure, but this is out of the scope of this study. The 9-1-1 rings are very similar in size and shape to the PCNA sliding clamp. X-ray structure studies of the Escherichia coli, phage T4, and eukaryotic PCNA sliding clamps have shown them to be nearly superimposable despite large differences in the protein primary structure and number of subunits (Refs. 20 and 21, and for review, see Ref. 27). These studies revealed a Christmas wreath-like ring with a 3.5–3.8-nm hole, an 8.5-nm diameter, and an ~3-nm height. To the resolution provided here, the 9-1-1 ring appears very close to this structure. Thus, these micrographs reveal that the checkpoint Rad proteins hRad9, hHus1, and hRad1 form a PCNA-like ring with potentially...
PCNA-like functions specialized for damaged DNA.

Structure of hRad17-RFC—Electron microscopic analyses of RFC (22) and the RFC functional homolog in archaea (28) as well as x-ray crystal structures of the bacterial (29) and archaeal (30) homologs demonstrate that this complex forms a more compact particle than the PCNA ring and depending on the angle of viewing (for review, see Ref. 27) would exhibit a deep groove giving it a U shape. Indeed, in the EM study (22), which used a glycerol spraying method similar to the one we used, RFC particles with this shape were the predominant form observed. The only difference between the RFC and hRad17-RFC complexes is that the RFC large subunit (p140) is replaced by the hRad17 protein (molecular mass, 75 kDa), and thus we would expect these complexes to form similar structures. Fig. 3 shows examples of the hRad17-RFC complex prepared in parallel with the checkpoint 9-1-1 complexes. Some particles (Fig. 3, A, arrow, and B, panels 4, 6, and 12) showed what appeared to be a small hole, but more frequently there was a deep groove that cut across at least half the diameter of the particle (Fig. 3B, panels 3, 5, 7, 9, and 11). The micrographs also suggest that these particles were able to lie on the EM support in a number of different arrangements indicative of a structure less asymmetric than the 9-1-1 ring. The hRad17-RFC complex has a molecular mass 2 times that of the checkpoint 9-1-1 complex. Comparison of the projected areas of the checkpoint 9-1-1 and hRad17-RFC complexes in parallel shadowed samples showed that the hRad17-RFC complexes were ~20–30% greater (n = 50 each). This is consistent with the hRad17-RFC particle being less asymmetric than the 9-1-1 ring but still not spherical as a spherical particle of 10-nm diameter would have a volume (hence mass) 3.5 times that of the flat 9-1-1 ring. We conclude that our images of hRad17-RFC particles are very similar to those of RFC described by Shiomi et al. (22) which in turn were consistent with the x-ray structures of these complexes (for review, see Ref. 27). Efforts were made to examine the complex of the 9-1-1 ring bound to the hRad17-RFC complex. Although some complexes consistent with these structures were seen, we also observed nonuniform aggregates, and thus these studies were postponed for the future.

Conclusions—Checkpoint Rad complexes play an essential role in the DNA damage checkpoint response. The existence of human complexes with structural similarity to the RFC/PCNA replication complexes has recently been suggested, but direct evidence for such similarity was lacking. Indeed the data were consistent with alternative models, and some of the data was not consistent with the ring-like structure for the checkpoint 9-1-1 complex (19). Here we show that the checkpoint 9-1-1 complex forms a flat ring with a distinct hole very similar to PCNA. hRad17-RFC complexes were visualized as more compact U-shaped particles closely resembling the structures described for RFC. Indeed the hRad17-RFC complex is likely dynamic with the capability of acting as a damage-sensing molecular machine where the subunits move in an ATP-dependent manner to open the checkpoint 9-1-1 complex and load it onto DNA.

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