

Requirement for ATP by the DNA Damage Checkpoint Clamp Loader*

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The DNA damage clamp loader replication factor C (RFC-Rad24) consists of the Rad24 protein and the four small Rfc2–5 subunits of RFC. This complex loads the heterotrimeric DNA damage clamp consisting of Rad17, Mec3, and Ddc1 (Rad17/3/1) onto partial duplex DNA in an ATP-dependent manner. Interactions between the clamp loader and the clamp have been proposed to mirror those of the replication clamp loader RFC and the sliding clamp proliferating cell nuclear antigen (PCNA). In that system, three ATP molecules bound to the Rfc2, Rfc3, and Rfc4 subunits are necessary and sufficient for efficient loading of PCNA, whereas ATP binding to Rfc1 is not required. In contrast, in this study, we show that mutant RFC-Rad24 with a *rad24-K115E* mutation in the ATP-binding domain of Rad24 shows defects in the ATPase of the complex and is defective for interaction with Rad17/3/1 and for loading of the checkpoint clamp. A similar defect was measured with a mutant RFC-Rad24 clamp loader carrying a *rfc4K55R* ATP-binding mutation, whereas the *rfc4K55E* clamp loader showed partial loading activity, in agreement with genetic studies of these mutants. These studies show that ATP utilization by the checkpoint clamp/clamp loader system is effectively different from that by the structurally analogous replication system.

Cell cycle arrest in response to DNA damage in an eukaryotic cell is mediated through a pathway, the DNA damage checkpoint, which is initiated by damage sensors and ultimately accomplished by the Cdc2 kinase that drives the cell cycle. One of the earliest steps in this pathway comprises the action of a clamp and a clamp loader that are structural homologs of the proliferating cell nuclear antigen (PCNA)¹ and replication factor C (RFC), respectively (reviewed in Refs. 1–3). The checkpoint clamp proteins Rad17, Mec3, and Ddc1 (Rad1, Hus1, and Rad9 in *Schizosaccharomyces pombe* and human) display sequence homology with each other and with PCNA, and this

complex has been purified as a heterotrimer from several sources (4–10). Similarly, the DNA damage checkpoint clamp loader consisting of *Saccharomyces cerevisiae* Rad24 (Rad17 in *S. pombe* and human) together with the four small Rfc2–5 subunits has been purified as a heteropentameric complex from several organisms (5–11). In this study, the checkpoint clamp is designated as Rad17/3/1 and the clamp loader is designated as RFC-Rad24.

DNA-independent interactions of the checkpoint clamp loader with its clamp have been studied. Rad17/3/1 and RFC-Rad24 interact in the absence of DNA (6, 9, 10). As previously observed with PCNA and RFC, the formation of a stable Rad17/3/1-RFC-Rad24 complex was strongly enhanced by ATP binding but did not require its hydrolysis (12). Loading of the Rad17/3/1 clamp around partial duplex DNA can be mediated by either ATP or ATP γ S; however, ATP hydrolysis was required to release the clamp from the clamp loader and permit sliding of Rad17/3/1 across double-stranded DNA (8, 10). The nature of the DNA substrate for this system has been a matter of discrepancy. We determined that yeast Rad17/3/1 is loaded onto partial duplex DNA with either 5'- or 3'-recessed termini and can even be loaded onto double-stranded DNA ends (10). However, loading on a normal template-primer substrate, *i.e.* with a 3'-recessed terminus, was most efficient. The human 9/1/1 clamp could be loaded onto DNA nicks as well as gapped DNA (9). In one study, a decided preference for 5'-recessed termini was measured (8), whereas the opposite preference was determined in another study (7).

In vivo, the single-stranded DNA-binding protein RPA is essential for recruitment of Rad17/3/1 to sites of DNA damage (7). *In vitro*, RPA was also required for efficient loading of the checkpoint clamp in some but not in other studies (7–10). The RPA dependence for clamp loading may be a reflection of the presence of extended single-stranded DNA regions in the substrate DNA. DNA substrates with very small gaps or nicks served as excellent loading templates in the absence of RPA (9, 10), whereas substrates with more extended regions of single-stranded DNA required RPA for loading (7).

In this paper, we have investigated the role of ATP in the checkpoint clamp-clamp loader system. Previous studies with RFC and PCNA have shown that the ATP-binding domains in Rfc2, Rfc3, and Rfc4 are both essential and sufficient for clamp loading. In contrast, the ATP-binding domain in Rfc1 is dispensable for clamp loading *in vitro* and *rfc1-K359E* mutants (with a mutation in the conserved lysine of the Walker A motif) show virtually no defects *in vivo* (13, 14). However, the analogous lysine \rightarrow glutamic acid mutation in the *RAD24* gene results in a complete loss of function phenotype for the DNA damage checkpoint (15). We have determined that ATP binding to Rad24 is essential for complex formation with the Rad17/3/1 clamp and the observed *in vivo* that defect can be directly interpreted as a failure to load the clamp.

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¹ The abbreviations used are: PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RFC-Rad24, complex of Rfc2–5 and Rad24; RFC-Rad24_{K115E}, complex with the Rad24K115E mutation; RFC_{4K55E}-Rad24, complex with the Rfc4K55E mutation; RFC_{4K55R}-Rad24, complex with the Rfc4K55R mutation; SS, single-stranded; ATP γ S, adenosine 5'-O-(3-thiotriphosphate); SPR, surface plasmon resonance; GST, glutathione S-transferase; PKA, protein kinase A; WT, wild type; RPA, replication protein A.

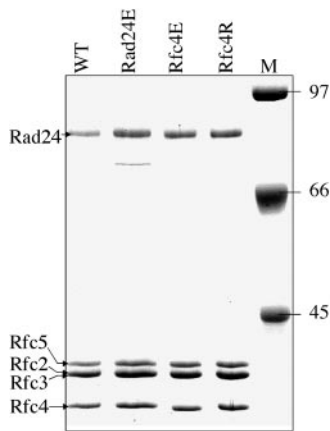


FIG. 1. Analysis of RFC-Rad24 mutants. 10 μ g of purified RFC-Rad24 (lane 1) and 15 μ g of RFC-Rad24K115E (lane 2), RFC_{4K55E}-Rad24 (lane 3), and RFC_{4K55R}-Rad24 (lane 4) were analyzed by 10% SDS-PAGE gel and stained with Coomassie Blue. M, molecular weight markers.

EXPERIMENTAL PROCEDURES

Plasmids and DNA—All plasmids for overexpression of wild-type RFC-Rad24 and Rad17/3/1 have been described previously (10). Plasmid pBL766-E (2 μ M ori *URA3 GST-rad24-K115E*) containing *rad24-K115E* was made by swapping a restriction fragment containing the mutation from plasmid YlpT-Rad24-K115E (a gift of Dr. Ken Sugimoto) into pBL766 (15). Plasmids pBL422-4R (2 μ M ori *URA3 RFC2 RFC3 rfc4-K55R RFC5*) and pBL422-4E (2 μ M ori *URA3 RFC2 RFC3 rfc4-K55E RFC5*) were made by swapping in appropriate restriction fragments from pBL410-R and pBL410-E, respectively (13, 14). For each plasmid, the region that had undergone restriction fragment swapping was completely sequenced to confirm that only the intended mutations were introduced.

The oligonucleotides used in the surface plasmon resonance (SPR) analysis and ATPase assays were A9 (5'-ccagtgaattcgagctcggtaccgctagcggggatctctca-3') and complementary oligonucleotides 5'-20 (5'-accgagctcgaattcactgg-3') and 3'-20 (5'-tagaggatcccctgtagcgg-3'). For attachment to SPR chips, the A9 oligonucleotide was biotinylated either at the 3' or 5' terminus and attached to streptavidin-coated chips (see below).

Expression and Purification of RFC-Rad24 Protein Complexes—For overproduction of RFC-Rad24K115E, *S. cerevisiae* strain BJ2168 (*MATa:ura3-52, trp1-289, leu2-3,112, prb1-1122, prc1-407, pep4-3*) was used. Yeast cells were co-transformed with plasmids pBL766-E and pBL422 and grown and induced in a 35-liter fermenter by using conditions and media described previously (10). After a 3-h induction with galactose, methylmethane sulfonate was added to a final concentration of 0.01% followed by a further 2 h of growth. As a host for RFC-Rad24-4E or RFC-Rad24-4R protein expression the strain PY94 (*Mata ade2-1, his3-11,15, ura3-1, trp1-1, leu2-3,112, can1-100, RFC4 Δ :kanMX6* with pBL 620 (Bluescript CEN ARS *RFC4 TRP1*)) was used. The strain was transformed with plasmid pBL422-RFC4R or pBL422-RFC4E, and transformants were grown for several generations on selective media for the pBL422 plasmids only. After plating on selective media, isolates that had lost pBL620 were readily identified. This indicates that expression of *rfc4-K55R* and *rfc4-K55E* from the galactose-inducible *GAL1-10* promoter on the multicopy plasmid was sufficient to permit growth, even though the cells were grown on glucose-containing media. These isolates were then transformed with plasmid pBL766 and grown and induced as described for the RFC-Rad24K115E complex described above.

RFC-Rad24 mutants were purified using a protocol described previously for the wild-type protein (10). The chromatographic properties and purity of each mutant complex were comparable to wild type, indicating that the mutations did not cause severe folding and/or protein-protein association problems (Fig. 1). The mutant RFC-Rad24 proteins (0.1–5 μ g) were analyzed by SDS-10% PAGE (Fig. 1) and stained with Sypro Red (Amersham Biosciences), and relative bands intensities were determined by phosphorimaging analysis. The molar ratio of Rfc5: (Rfc2 + Rfc3):Rfc4 (Rfc2 and Rfc3 were poorly separated) were 1.2:1 with <10% variation from complex to complex. The molar ratio of Rad24 to Rfc2–5 was 0.9:1 for all of the complexes.

A ³²P-labeled Form of Rad17/3/1—The *RAD17* gene in plasmid

pBL764 was modified at the BamHI site by insertion of an oligonucleotide specifying the MLRRASVGS peptide sequence preceding the natural methionine codon of the *RAD17* gene, giving plasmid pBL764-PKA. The modified clamp (Rad17_{PKA}/3/1) was purified from a yeast overexpression system as described previously (10). Rad17_{PKA}/3/1 (10 μ g) was labeled in a 30- μ l reaction with 50 μ Ci of carrier-free [γ -³²P]ATP and 5 units of cAMP-dependent protein kinase (catalytic subunit) for 30 min at 30 °C according to manufacturer's instructions (New England Biolabs). The factor was purified from unincorporated radioactivity by G25 gel filtration.

Analysis of the Checkpoint Complexes and Their Interactions—Glycerol gradient centrifugation of 25 μ g of wild-type or mutant RFC-Rad24 with or without 25 μ g of Rad17/3/1 was performed in a 5-ml 10–30% glycerol gradient in buffer C (25 mM Hepes-NaOH, pH 7.8, 100 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol) containing either 1 mM ATP or 50 μ M ATP γ S in an SW65 rotor (Beckman) at 4 °C for 20 h at 50,000 rpm. The protein in 180- μ l fractions was precipitated with 10% trichloroacetic acid and analyzed by SDS-10% PAGE. Carbonic anhydrase (2.8 S), bovine serum albumin (4.3 S), alcohol dehydrogenase (7.4 S), β -amylase (8.9 S), catalase (11.3 S), and ferritin (17.6 S) were used as sedimentation markers.

ATPase Assays—Assays of 25 μ l were performed in buffer C supplemented with 50 μ g/ml bovine serum albumin. The assays contained 50 nM RFC-Rad24, 100 nM Rad17/3/1 or PCNA, 50 μ M [α -³²P]ATP, and 1 μ M effector DNA. After 2.5, 5, 7.5, and 10 min at 30 °C, 5- μ l aliquots were removed from the reaction and quenched with 2 μ l of 50 mM EDTA, 1% (w/v) SDS, and 20 mM each ADP and ATP. 2 μ l were spotted on a polyethyleneimine cellulose sheet and dried. The sheet was washed in water for 10 min, rinsed in ethanol, dried, and developed in 0.5 M LiCl/1 M formic acid. The sheet was dried and subjected to PhosphorImager analysis.

DNA-Protein Interaction Analysis—Surface plasmon resonance was performed in a BIAcore X apparatus. Buffer C containing 100 μ g/ml bovine serum albumin was the running buffer used in the analysis. DNA chips were prepared as described and contained ~50–100 resonance units of biotinylated DNA attached via streptavidin to a CM5 chip. The interaction between RFC-Rad24 mutants and Rad17/3/1 with DNA was monitored at 20 °C by injecting, at a flow rate of 30 μ l/min, 80 μ l of the protein factors and ATP or ATP γ S at the indicated concentration over a DNA chip. The dissociation constant of the ATP-dependent DNA-RFC-Rad24-Rad17/3/1-binding reaction was determined by plotting the initial binding rates against the ATP concentration and fitting the data to a simple Langmuir binding model: $V_{max} = [ATP]/(K_D + [ATP])$.

Gel Filtration Analysis of Clamp Loading—150 fmol of double-stranded circular Bluescript DNA with a single-stranded gap of 82 nucleotides (at the XhoI-SacII sites) was incubated with 250 fmol of wild-type or mutant RFC-Rad24 and 2 pmol of [³²P]Rad17/3/1 in buffer A (25 mM Hepes-NaOH, pH 7.8, 75 mM NaCl, 8 mM MgCl₂, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin) and varying ATP concentrations at 23 °C for 3 min and then loaded onto a 5-ml BioGel A5m column equilibrated in buffer A. The radioactivity in each 6-drop fraction was determined in a scintillation counter.

RESULTS

Complex Formation between RFC-Rad24 and Rad17/3/1—RFC-Rad24 forms a stable equimolar complex with Rad17/3/1, which can be isolated by gel filtration or glycerol gradient centrifugation, but only if the complex is formed in the presence of ATP or the non-hydrolyzable analog ATP γ S (10). Rad17/3/1 alone does not bind DNA, and RFC-Rad24 shows weak binding to DNA in the presence of ATP. Therefore, the strong interaction with DNA observed when RFC-Rad24, Rad17/3/1, and ATP are present must indicate that RFC-Rad24 forms an ATP-dependent complex with Rad17/3/1 prior to interaction with DNA (10). This property should allow us to quantitatively determine the ATP-dependent interaction between RFC-Rad24 and Rad17/3/1 indirectly by measuring DNA binding in a quantitative manner.

We carried out a series of titration experiments in which protein binding to partial duplex DNA was measured by SPR. The interaction between the checkpoint factors and DNA was monitored in the presence of saturating ATP or ATP γ S (see also Fig. 5) by injection of low and constant concentration (3 nM) of one complex and increasing concentrations of the other

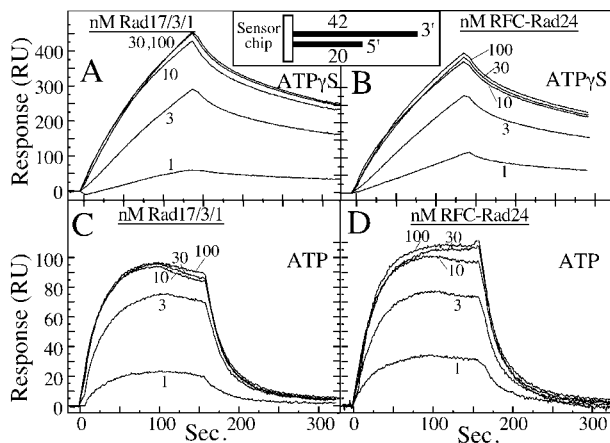


FIG. 2. Quantitation of interactions between RFC-rad24 and Rad17/3/1. The binding of RFC-Rad24 and Rad17/3/1 to DNA was studied using a 5'-recessed DNA substrate attached to a BIAcore CM5 via a biotin-streptavidin linkage (see *inset*). A–D, the checkpoint factors were injected at a constant 3 nM concentration of RFC-Rad24 and increasing concentrations of Rad17/3/1 (A and C) or at a constant 3 nM concentration of Rad17/3/1 and increasing concentrations of RFC-Rad24 (B and D) and either 100 μ M ATP γ S or 1 μ M ATP. RFC-Rad24 with Rad17/3/1 but no nucleotide showed a response of <3 response units (R.U.). RFC-Rad24 without Rad17/3/1 but with ATP or ATP γ S showed a response of <10 R.U. These curves have been subtracted in the figure.

factor. Fig. 2, panels A and C, shows binding curves obtained at a constant concentration of the RFC-Rad24 clamp loader and increasing concentrations of the Rad17/3/1 clamp in the presence of saturating ATP γ S or ATP, respectively, whereas panels B and D represent binding isotherms at a constant concentration of Rad17/3/1 complex and increasing concentration of RFC-Rad24. Similar shape and response amplitude for each pair of the corresponding binding curves not only confirms that the checkpoint factors form a complex prior to DNA binding but also allows us to make a quantitative determination of the K_D value of the RFC-Rad24-Rad17/3/1 complex of 2.5 ± 0.5 nM, either with ATP or ATP γ S present. This K_D value compares well with that of 1.3 nM for the RFC-PCNA complex in the presence of ATP or ATP γ S, which was determined under the same solution conditions (16). All of the additional kinetic experiments in this study used 50 nM RFC-Rad24 and 100 nM Rad17/3/1 to ensure extensive complex formation between clamp loader and clamp.

Purification of RFC-Rad24 Mutants—To further investigate the role of the ATP-binding domains in RFC-Rad24 for checkpoint clamp loader function, we purified three mutant complexes with mutations in the conserved lysine of the Walker A motif (17). The *rad24-K115E* mutation results in a complete loss of function phenotype (15). A K55R mutation in the *RFC4* gene displays a mild checkpoint defect, whereas the K55E mutant shows no phenotype (14). Additionally, a *rfc2-K71R* mutation shows a checkpoint defect but the very poor growth of yeast cells containing this mutation, particularly when *RAD24* was overexpressed, prohibited cell growth for biochemical purposes (results not shown). The mutant complex containing Rad24-K115E was overexpressed in a wild-type yeast background because the presence of the GST tag on Rad24-K115E allowed it to be purified away from chromosomally expressed wild-type Rad24. However, the complexes with mutant Rfc4 were overexpressed in a *RFC4* deletion background (see “Experimental Procedures” for details).

The mutant complexes were purified by glutathione-affinity chromatography followed by viral protease treatment to remove the GST domain and finally by Mono S chromatography as described previously for the wild-type complex (10).

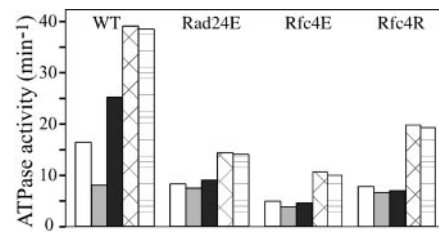


FIG. 3. ATPase activity of the RFC-Rad24 mutants. The assays contained, when present, 50 nM RFC-Rad24, 100 nM Rad17/3/1 or PCNA, 50 μ M [α - 32 P]ATP, and 1 μ M 3'-recessed DNA (A9/3'-20). Percentage of ADP formed was quantitated by PhosphorImager analysis and used to calculate the ATPase activity (expressed as turnover number per min). White, RFC-Rad24 alone; gray, RFC-Rad24 + Rad17/3/1; black, RFC-Rad24 + Rad17/3/1 + DNA; cross-hatched, RFC-Rad24 + PCNA; lined, RFC-Rad24 + PCNA + DNA.

Similarly, to the wild-type RFC-Rad24 complex, all three mutants formed approximately stoichiometric complexes containing equimolar amounts of Rad24 and each of the four Rfc2–5 subunits (Fig. 1) (see also “Experimental Procedures”). The Rfc4K55E subunit in the mutant complex migrated slightly faster than wild-type Rfc4 (13). The proper subunit stoichiometry of the complexes together with their conserved chromatographic properties suggest that the mutations did not substantially alter folding and/or stability of the affected subunits.

ATPase Activity of the RFC-Rad24 Mutants—All of the mutant complexes showed ATPase activity. The ATPase activity of the mutants, however, was reduced to ~30–60% of that of wild type (Fig. 3). The ATPase of wild-type RFC-Rad24 is significantly inhibited in the presence of Rad17/3/1 but stimulated when both the clamp and substrate DNA are present, indicative of active clamp loading (10). However, the ATPase activity of all three mutants studied is neither significantly decreased by the presence of the checkpoint clamp nor increased by the clamp together with effector DNA, suggesting a defect in the interaction with the clamp and in clamp loading.

Our earlier study revealed that the ATPase activity of RFC-Rad24 is strongly stimulated by PCNA (~2.4-fold), yet the ATPase is not further stimulated when effector DNA is also present, suggesting an interaction between the two complexes but one that does not lead to productive clamp loading (Fig. 3) (10). We observed that the mutant complexes were also stimulated by PCNA (~1.5–2-fold), but no further stimulation was observed when DNA was also present. Therefore, ATP binding to both the Rad24 and Rfc4 subunits is essential for inhibition of the ATPase by Rad17/3/1 and for stimulation of the ATPase by both Rad17/3/1 and DNA; however, the apparently transient interactions between RFC-Rad24 and PCNA do not require an intact ATP-binding domain in the Rad24 subunit and even occur when the ATP-binding domain in Rfc4 is mutated as observed previously (13).

ATP-dependent Complex Formation between RFC-Rad24 and Rad17/3/1—The replication clamp loader RFC efficiently binds and loads PCNA onto DNA even with an inactivating K359E mutation in the conserved lysine of the ATP-binding site of Rfc1 (13). To test whether RFC-Rad24 also does not require ATP binding to the large subunit Rad24 for interaction with its clamp, we conducted a velocity sedimentation analysis to analyze complex formation. Contrasting to the wild-type clamp loader, RFC-Rad24K115E failed to form a detectable complex with Rad17/3/1 in the presence of 1 mM ATP or 100 μ M ATP γ S (Fig. 4D, data shown only for ATP γ S). The K55R and K55E mutations in the ATP binding motif of Rfc4 also caused a failure to detect a complex by glycerol gradient centrifugation between mutant RFC-Rad24 and Rad17/3/1 in the presence of 1 mM ATP or 100 μ M ATP γ S (data not shown). These data indicate that a stable ATP-dependent complex between RFC-

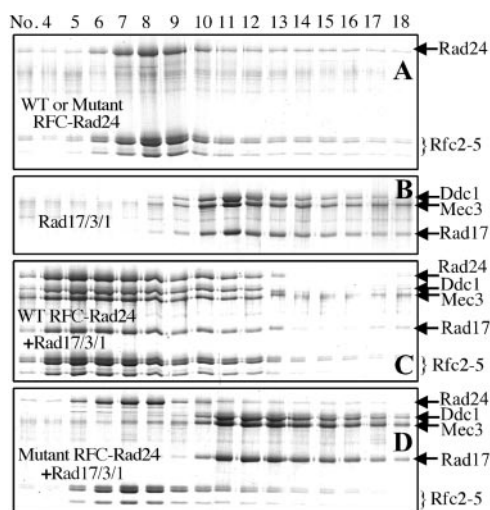


FIG. 4. Isolation of RFC-Rad24-Rad17/3/1 complexes. RFC-Rad24 and Rad17/3/1 were centrifuged separately (A and B, respectively) through a glycerol gradient. C, a mixture of RFC-Rad24 and Rad17/3/1 together with 1 mM ATP. D, a mixture of RFC-Rad24K115E and Rad17/3/1 together with 1 mM ATP. Fractions were collected from the bottom of the gradient, precipitated with 10% trichloroacetic acid, and analyzed by 10% SDS-PAGE (See “Experimental Procedures” for details). Not shown, all three mutant clamp loaders centrifuged separately gave the same pattern as wild type (A). The Rfc4K55E and Rfc4K55R complexes centrifuged together with Rad17/3/1 and 1 mM ATP gave the same pattern as in D. Substituting 100 μ M ATP γ S for 1 mM ATP yielded the analogous centrifugation patterns.

Rad24 and Rad17/3/1 clamp can only be formed when the ATP-binding sites of both Rad24 and Rfc4 are functional.

Defective Clamp Loading by Mutant RFC-Rad24—The velocity sedimentation experiments (Fig. 4) revealed that mutant RFC-Rad24 does not form an isolatable complex with Rad17/3/1. However, transient complexes might still be formed, which could be competent for loading onto partial duplex DNA. To test this hypothesis, we used SPR to detect loading. In this assay, a 3'-recessed partial duplex DNA substrate was used to which the wild type RFC-Rad24-Rad17/3/1 system had been shown to display the highest affinity (Fig. 5) (10). The mutant RFC-Rad24_{K115E} complex was completely defective for loading Rad17/3/1 onto this DNA substrate, either with 1 mM ATP or 100 μ M ATP γ S (Fig. 5B). Surprisingly, clamp loader complexes containing the Rfc4 ATP-binding domain mutants K55R and K55E, which did not appear to form stable complexes with Rad17/3/1, were able to load Rad17/3/1 onto the DNA albeit with much lower efficiency than wild type (Fig. 5, A, C, and D). In this assay, the mutant Rfc4K55E complex showed a substantial higher activity than the Rfc4K55R complex.

To assess the quantitative requirement for ATP in the formation of a stable RFC-Rad24-Rad17/3/1 complex, we varied the ATP concentration in the SPR experiment. The linear initial rate of binding to the DNA chip is dependent on the concentration of active complexes, *i.e.* those RFC-Rad24 molecules that have the required number of ATP molecules bound to form both a high affinity complex with Rad17/3/1 and load Rad17/3/1 onto the DNA on the chip. Therefore, by varying the ATP concentration at constant concentrations of RFC-Rad24 and Rad17/3/1, the effective K_D for clamp loading can be determined from the initial binding rates. The data points were fitted to a simple Langmuir binding model: $v_{in} = [ATP]/(K_D + [ATP])$. The half-maximal response for wild-type RFC-Rad24 was obtained at 5 μ M ATP (Fig. 6). Because multiple ATP binding events are involved in clamp loading, this K_D may represent the binding of an ATP molecule at the lowest affinity site. Assuming that a similar maximal initial binding rate to the chip occurs with the

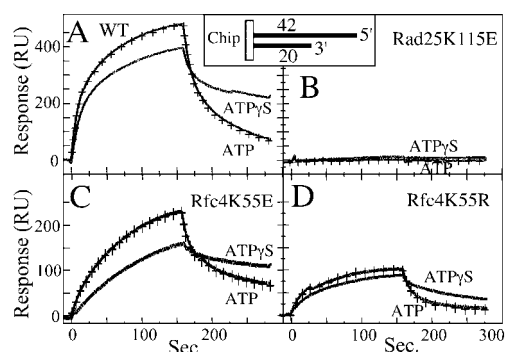


FIG. 5. DNA loading of Rad17/3/1 by mutant RFC-Rad24. The loading of the Rad17/3/1 clamp by RFC-Rad24 complexes was studied by SPR using a 3'-recessed DNA substrate (see inset). 50 nM RFC-Rad24 or the indicated mutant and 100 nM Rad17/3/1 were injected in the presence of either 1 mM ATP (crossed line) or 100 μ M ATP γ S (solid line). Note the different y axis scales for the experiments in A and B and those in C and D. For details, see “Experimental Procedures” and the legend to Fig. 2.

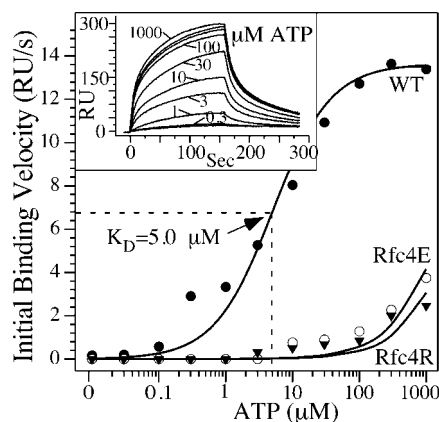


FIG. 6. Quantitation of ATP requirement in the checkpoint clamp loading reaction. Loading of Rad17/3/1 by wild-type and mutant RFC-Rad24 was analyzed exactly as described in Fig. 5. However, the injections were carried out in the presence of increasing ATP concentrations (from 0.01 to 1000 μ M). The inset shows the response curves obtained for wild-type RFC-Rad24. The initial binding rates were plotted against the ATP concentration and fitted to the simple Langmuir binding model as described under “Experimental Procedures.”

Rfc4K55R and Rfc4K55E mutant clamp loaders at saturating ATP, the K_D value was estimated at \sim 2 mM for the Rfc4K55E mutant and \sim 3 mM ATP for the Rfc4K55R mutant loader.

One disadvantage of the SPR system is that, by necessity, rather small oligonucleotides need to be employed to detect a signal. To determine whether the SPR analysis accurately reflects the loading of Rad17/3/1 at template-primer junctions with extended regions of double-stranded DNA that permit sliding of the clamp, we also carried out a more classical loading assay on gapped circular DNA molecules. For purposes of quantitation, a phosphorylatable (PKA) tag was fused to the N terminus of Rad17 and the complex was labeled with 32 P to a specific radioactivity of \sim 50,000 cpm/pmol (see “Experimental Procedures”). Loading of Rad17/3/1 was detected by analysis of the loading mixture over a gel filtration column, which separates DNA-bound clamp in the void volume from unbound protein (Fig. 7A).

Loading of the checkpoint clamp was dependent on the presence of both RFC-Rad24 and ATP (data not shown). Under the conditions used, 400–500 fmol of Rad17/3/1 were retained with 150 fmol of gapped circular DNA, indicating that multiple clamps can be loaded (Fig. 7A). In contrast, in the presence of the non-hydrolyzable analog ATP γ S, the loading of only one clamp per DNA molecule was observed. These results indicate

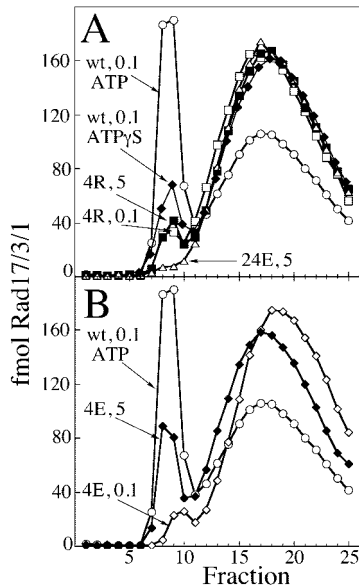


FIG. 7. Loading of Rad17/3/1 onto gapped circular DNA. *A*, loading of ^{32}P -labeled Rad17/3/1 by wild-type RFC-Rad24 with either 0.1 mM ATP (*wt*, 0.1 ATP) or 0.1 mM ATP γ S (*wt*, 0.1 ATP γ S), RFC-Rad24_{K115E} with 5 mM ATP (24E, 5), and RFC_{455E}-Rad24 with either 0.1 mM ATP (4R, 0.1) or 5 mM ATP (4R, 5). *B*, loading of wild type (as in *A*) and RFC_{455E}-Rad24 with either 0.1 mM ATP (4E, 0.1) or 5 mM ATP (4E, 5). The DNA-protein complex elutes in fractions 7–10 and free protein in fractions 12–25. See “Experimental Procedures” for details.

that upon termination of clamp loading, hydrolysis of ATP promotes dissociation of RFC-Rad24 and sliding of Rad17/3/1 away from the loading point and allows additional clamps to be loaded (10).

RFC-Rad24_{K115E} was completely inactive in loading Rad17/3/1, even with 5 mM ATP present. RFC_{4K55R}-Rad24 also showed very poor loading efficiency, but surprisingly, the signal we observed was not substantially increased by increasing the ATP concentration from 0.1 to 5 mM. In contrast, RFC_{4K55E}-Rad24 showed poor loading efficiency at 100 μM ATP; however, at 5 mM ATP, 150–200 fmol of Rad17/3/1 was associated with 150 fmol of gapped DNA (Fig. 7*B*).

DISCUSSION

Recent studies of the eukaryotic replication clamp loading system have shown that clamp loading is a sequential ordered process in which formation of a RFC-PCNA complex precedes binding to the DNA substrate (18). Each successive step on the reaction pathway is propagated by the binding of additional ATP to RFC. Initially, ATP binding to RFC is followed by binding of PCNA. The formation of this complex induces a conformational change, which makes one additional ATP-binding site available. Upon binding to DNA of this PCNA-RFC-ATP complex, another conformational change in RFC makes one final ATP-binding site available. This final ATP needs to be bound in order for the loading process to proceed to completion. The functional significance of these ATP binding events was addressed by a mutational analysis of the ATP-binding domains in the Rfc1–4 subunits. This analysis showed that, whereas binding of ATP to Rfc2, Rfc3, and Rfc4 is essential for clamp loading to occur, binding to Rfc1 appears to be dispensable. The ATP K_D values for PCNA loading by wild-type RFC and Rfc1K359E-RFC were virtually identical, 6.7 and 8.4 μM ATP, respectively (Table I) (13).

This study was undertaken to gain a better understanding of the checkpoint clamp loader/clamp interactions. Are the interactions of RFL-Rad24 with Rad17/3/1 directly analogous to the RFC-PCNA system, or do specialized interactions exist not just

TABLE I

Comparative analysis of the replication and checkpoint clamp loaders

Data for RFC-PCNA were taken from Refs. 13 and 16. NA, not applicable; ND, not done.

Clamp loader-clamp	RFC-PCNA	RFC-Rad24-Rad17/3/1
K_D of clamp loader-clamp complex ^a		
Wild-type	1.3 \pm 0.4 nM	2.5 \pm 0.5 nM
Rfc1K359E	4 \pm 2 nM ^b	NA
Rad24K115E	NA	No complex ^d
Rfc4K55E	3 \pm 1 nM ^b	No complex ^d
Rfc4K55R	ND	No complex ^d
ATP K_D for clamp loading		
Wild-type	6.7 \pm 1 μM ^c	5.0 \pm 1 μM
Rfc1K359E	8.4 \pm 1 μM ^c	NA
Rad24K115E	NA	No loading
Rfc4K55E	90 \pm 10 μM ^c	2.2 \pm 1 mM
Rfc4K55R	ND	3.4 \pm 1 mM

^a In the presence of 1 mM ATP, all of the measurements were by SPR.

^b X. V. Gomes and P. M. Burgers, unpublished results.

^c In the presence of 1 mM ATP, all of the measurements were by coupling PCNA loading to replication of poly(dA)-oligo(dT) by DNA polymerase δ and dTTP.

^d In the presence of 1 mM ATP, all of the measurements were by glycerol gradient centrifugation.

between the checkpoint clamp and Rad24 but also with the small subunits of RFC? In particular, it was of interest to determine whether the known absolute defect in checkpoint response of the *S. cerevisiae rad24-K115E* mutant was due to its failure to load the clamp or to another step in the checkpoint response pathway. Although we cannot exclude that ATP binding to Rad24 may have a second function in checkpoint response, the observed genetic phenotype can be explained by the failure of the mutant complex to load Rad17/3/1 onto effector DNA substrate.

Previous studies of Walker A motif mutations in Rfc4 presented an interesting deviation from the accepted rule that conservative Lys \rightarrow Arg mutations show less severe phenotypes than the charge-reversal Lys \rightarrow Glu mutations. Whereas mutations in Rfc2 and Rfc3 follow this pattern, it is reversed in Rfc4 (13, 14). *In vivo*, the *rfc4-K55R* mutant showed mild growth defects and a partial checkpoint defect. All of these phenotypes were absent in the *rfc4E* mutant. Structural analysis of several ATP-binding proteins with mutations in this conserved lysine indicate at most slight structural alterations, and therefore, biochemical or genetic defects associated with mutations in this residue have in general been ascribed to defects in ATP binding and/or hydrolysis, rather than to defects in protein folding, protein stability, or association with other subunits in a complex (19–21).

The properties of the RFC_{455E}-Rad24 loader agree with our concept that its defect is caused by a defect in ATP binding and/or hydrolysis. Clamp loading by RFC_{455E}-Rad24 is quite poor at low concentrations of ATP. However, this defect is largely suppressed at very high concentrations of ATP, an indication that the mutation conferred a strong K_D defect for ATP binding (Fig. 7*B*). However, because the intracellular concentration of ATP is also estimated to be very high (\sim 2 mM), it appears likely that a partial activity of this mutant loader *in vivo* is sufficient to confer full checkpoint function (22). In contrast, the RFC_{4K55R}-Rad24 clamp loader shows poor activity at both low and high concentrations of ATP, suggesting additional defects in this mutant (Fig. 7*A*).

Therefore, a tentative conclusion of these studies is that the efficiency of the DNA damage checkpoint function in the cell is directly related to the efficiency of loading the Rad17/3/1 clamp by mutant RFC-Rad24. However, this simple argument ignores the complexity of interactions of the Rfc2–5 core, which can function in at least four distinct clamp loaders typified by

RFC1, *RAD24*, *CTF18*, and *ELG1* (23–27). Mutations in the latter two genes directly or indirectly affect DNA damage response and checkpoint function, and it is possible that the observed phenotypic defects of the *RFC4* mutations carry additional contributions from possible defects in the function of the *CTF18* and *ELG1* clamp loaders (26–28).

The defects in loading by the mutant clamp loaders may primarily be a consequence of the observed defect in their interaction with the Rad17/3/1 clamp (Fig. 4). It is this aspect of our mutational studies that shows the greatest differences between the replication and the checkpoint clamp loading system. Mutant forms of RFC with single ATP-binding site mutations in Rfc1, Rfc2, Rfc3, or Rfc4 (Rfc5 does not have a functional ATP-binding domain) form ATP-dependent complexes with PCNA with remarkably comparable efficiencies (Table I) (13). In contrast, none of the mutant RFC-Rad24 loaders forms a sufficiently stable complex with Rad17/3/1 to allow their co-migration by glycerol gradient centrifugation (Fig. 3). We have not yet made a SPR chip with functional Rad17/3/1 attached to measure interactions more quantitatively.

ATP serves a role in stabilizing interactions between the clamp loader and the clamp and between the clamp loader-clamp complex and the effector DNA and, finally, in promoting an ill-defined but required step after formation of the ternary clamp loader-clamp-DNA complex to complete the loading sequence. This study has highlighted obvious qualitative differences between the replication and the checkpoint system in ATP requirement. Yet, quantitative differences exist as well. The Rfc4K55E mutation raised the K_D value for ATP for PCNA loading by RFC to 90 μM from 6.7 μM for wild type. In contrast, the same mutation raised the K_D value for ATP for Rad17/3/1 loading by RFC-Rad24 to 2.2 mM from 5 μM for wild type (Table I). If the measured K_D value represents binding of ATP to that subunit with the lowest ATP binding affinity and this would obviously be Rfc4 in the mutant complex, it follows that different conformational constraints and structural changes are imposed upon the two clamp loaders during the binding of the clamp and during loading.

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