Direct evidence that a conserved arginine in RuvB AAA$^+$ ATPase acts as an allosteric effector for the ATPase activity of the adjacent subunit in a hexamer

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The *Escherichia coli* RuvA and RuvB protein complex promotes branch migration of Holliday junctions during recombinational repair and homologous recombination and at stalled replication forks. The RuvB protein belongs to the AAA$^+$ (ATPase associated with various cellular activities) ATPase family and forms a hexameric ring in an ATP-dependent manner. Studies on the oligomeric AAA$^+$ class ATPases suggest that a conserved arginine residue is located in close proximity to the ATPase site of the adjacent subunit and plays an essential role during ATP hydrolysis. This study presents direct evidence that Arg-174 of RuvB allosterically stimulates the ATPase of the adjacent subunit in a RuvB hexamer. RuvBR174A shows a dominant negative phenotype for DNA repair *in vivo* and inhibits the branch migration catalyzed by wild-type RuvB. A dominant negative phenotype was also observed with RuvBK68A (Walker A mutation). RuvB K68A–R174A double mutant demonstrates a more severe dominant negative effect than the single mutants RuvB K68A or R174A. Moreover, although RuvB K68A and R174A are totally defective in ATPase activity, ATPase activity is restored when these two mutant proteins are mixed at a 1:1 ratio. These results suggest that each of the two mutants has distinct functional defects and that restoration of the ATPase activity is brought by complementary interaction between the mutant subunits in the heterohexamers. This study demonstrates that R174 plays an intermolecular catalytic role during ATP hydrolysis by RuvB. This role may be a general feature of the oligomeric AAA/AAA$^+$ ATPases.

Holliday junctions are DNA structures in which two homologous duplex DNA molecules are held together by single-stranded DNA crossover. Holliday junctions are formed as reaction intermediates during genetic recombination, recombinational repair, and DNA replication. In *Escherichia coli*, Holliday junctions are processed into reaction products by RuvA, RuvB, and RuvC (1, 2). RuvA, which forms a symmetric tetrameric multimer and binds specifically to Holliday junctions (3, 4), forms a complex with RuvB and facilitates loading of RuvB onto DNA (5). The RuvAB complex promotes branch migration of the Holliday junction using the energy of ATP hydrolysis (6, 7). RuvC endonuclease is a dimer that cleaves the Holliday junction symmetrically at the crossover point (8, 9).

The multifunctional RuvB protein forms a hexameric ring that actively promotes branch migration in an ATP-dependent manner (1, 2). RuvB ATPase activity is strongly and synergistically enhanced by RuvA and DNA (7, 10, 11). Structural studies of *Thermus thermophilus* HB8 and *Thermotoga maritima* RuvB show that RuvB has a crescent-like shape with three domains (12, 13). The first two domains are characteristic of the AAA$^+$ ATPase protein family with conserved Walker A/B and sensor I/II motifs. These domains provide the ATPase catalytic center and mediate ATP-dependent conformational change (14). The C-terminal domain is similar to that of the winged helix DNA binding motif.

Arg-174 of RuvB lies between the sensor I and sensor II motifs and is highly conserved in all RuvB homologs (15). In a previous study, we characterized a RuvB Arg174His mutant that is defective during *in vivo* DNA repair (15). X-ray crystallographic studies suggested that Arg-174 lies close to the γ-phosphate of ATP bound to the adjacent subunit in the RuvB hexamer (12, 13), and the corresponding residue in other oligomeric AAA$^+$ or AAA ATPases is structurally similar (16–20). Furthermore, mutants of the corresponding arginine in FtsH and HslU are also ATPase-deficient (18, 21, 22). Previous studies identified an ''arginine finger'' in guanosine triphosphatase (GTPase)-activating proteins (GAPs) that may stimulate the GTP-binding protein (Ras or Rho) with which the GAP interacts. In that case, the conserved arginine of the GAP approaches the active site of the GTPase, which regulates GTPase activity by stabilizing the transition state of the hydrolytic reaction (23–26). RuvB Arg-174 may be an equivalent arginine finger with respect to the RuvB ATPase.

This study characterizes the mechanism of RuvB ATPase using mutant heterohexamers composed of wild-type, R174A, K68A, or K68A–R174A RuvB proteins. The results provide direct evidence that RuvB Arg-174 allosterically stimulates the ATPase activity of the adjacent subunit in hexameric RuvB.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* HRS2301 (AB1157Δ*ruvB*100::*cat*) and HRS4000 (BL21(DE3)-Δ*ruvABC*100::*kan*) were constructed by P1 transduction as described previously (27, 28) and were used for complementation tests and for protein overexpression, respectively. The expression vector pAF101 was described previously (29).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out by PCR using the appropriate synthetic 24-mer oligonucleotides and pRB100 (wild-type RuvB), a derivative of pAF101 (15). The following specific changes were engineered: codon 174 of *ruvB* from CGT (Arg) to GCA (Ala) produced plasmid pRB174; codon 68 of *ruvB* from AAA (Lys) to GCA (Ala) produced pRB68. pRB601 was constructed by replacing the 250-bp *Nde*I–*Xho*I fragment of pRB100 with the 250-bp *Nde*I–*Xho*I fragment from pRB68 (K68A). pRB602 was constructed by replacing the 250-bp *Xho*I–*Eco*RI fragment of pRB100 with the 250-bp *Xho*I–*Eco*RI fragment from pRB174 (R174A). To construct the K68A–R174A double mutant plasmid, the 250-bp *Nde*I–*Xho*I fragment of pRB601 was replaced by the 250-bp

Abbreviation: GTPase, guanosine triphosphatase.

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*Nde*I–*Xho*I fragment of pRB602 to produce pRB603. The DNA sequences of the mutant *ruvB* genes were confirmed by sequencing the appropriate DNA regions with an Applied Biosystems 373S DNA sequencer.

UV Sensitivity Test. The sensitivity of exponentially growing cells to UV irradiation was measured as described previously (27).

Enzymes. RuvA and RuvB proteins were purified as previously described (28).

Branch Migration Assay. The ATP-dependent branch migration activity of the RuvA–RuvB complex was assayed by measuring dissociation of synthetic Holliday junctions. Synthetic Holliday junctions were constructed by annealing four 72-mer deoxyoligonucleotides (JY11, JY12, JY13, and JY14) (27). The standard reaction mixture (20 μl) contained 20 mM Tris-acetate (pH 8.0), 10 mM $Mg(OAc)_2$, 1 mM DTT, 1 mM ATP, 0.01% (wt/vol) BSA, 5 nM ^{32}P-labeled synthetic Holliday junction DNA, 50 nM RuvA, and the indicated amount of RuvB. Reactions were initiated by addition of mutant or wild-type RuvB, incubated at 37°C for 30 min, and terminated by the addition of 5 μl of stop buffer (50 mM EDTA/5 mg/ml proteinase K/2% SDS). The products were analyzed by 9% polyacrylamide gel electrophoresis and visualized with a phosphorimager (Fuji BAS 1500).

ATPase Assays. ATP hydrolysis was measured as described previously (27). Wild-type RuvB (0.5 μM) and various amounts of mutant RuvB were premixed in the absence of ATP and $Mg(OAc)_2$. The reaction mixture (25 or 50 μl) contained 20 mM Tris-acetate (pH 8.0), 10 mM $Mg(OAc)_2$, 1 mM DTT, the indicated concentration of ATP, 3 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP, 0.01% (wt/vol) BSA, synthetic Holliday junction (100 nM in Holliday junction moles), and 0.6 μM RuvA protein. Reactions were initiated by the addition of RuvB and incubated at 37°C. Aliquots (5 μl) were sampled at the indicated times and immediately mixed with 5 μl of stop buffer (25 mM EDTA/5 mM ADP).

Results and Discussion

Mutation of Arginine Finger of RuvB. Arg-174 in *E. coli* RuvB is highly conserved among *ruvB* homologs (15). Structural analysis suggests that R174 is not in the catalytic region of the monomer unit of RuvB; however, R174 is catalytically important because it lies close to (or inserts into) the ATP-binding region (ATPase site) of the adjacent subunit of the RuvB hexamer (Fig. 1*A*) (12, 13). The RuvB hexamer is an ATPase that uses the coordinated energy of ATP hydrolysis by the hexamer to promote branch migration of Holliday junctions. Here, this process was studied by using two RuvB mutants: RuvB R174A mutant, which has a dominant negative phenotype *in vivo* for DNA repair (Fig. 1*B*) and is completely deficient in ATPase and branch migration *in vitro* (Fig. 1 *C* and *D*) (13, 15); and RuvB K68A (mutated in the Walker A motif), with a phenotype similar to R174A (see below) (30).

Effect of R174A on RuvAB-Catalyzed Branch Migration. The dominant negative phenotype of RuvB R174A implies that substitution of alanine for arginine at position 174 interferes with wild-type RuvB function. Previous studies showed that a mixture of RuvB K68A and wild-type RuvB forms inactive heterohexamers and therefore exerts a dominant negative phenotype for UV damage repair *in vivo* and branch migration activity *in vitro* (30). Here, ATPase and branch migration were examined when R174A, K68A, or K68A–R174A mutant RuvB were incorporated into a wild-type RuvAB complex. Fig. 2*A* shows dose-dependent inhibition of RuvAB-catalyzed branch migration by mutant RuvB subunits. Branch migration was inhibited by 50% when the

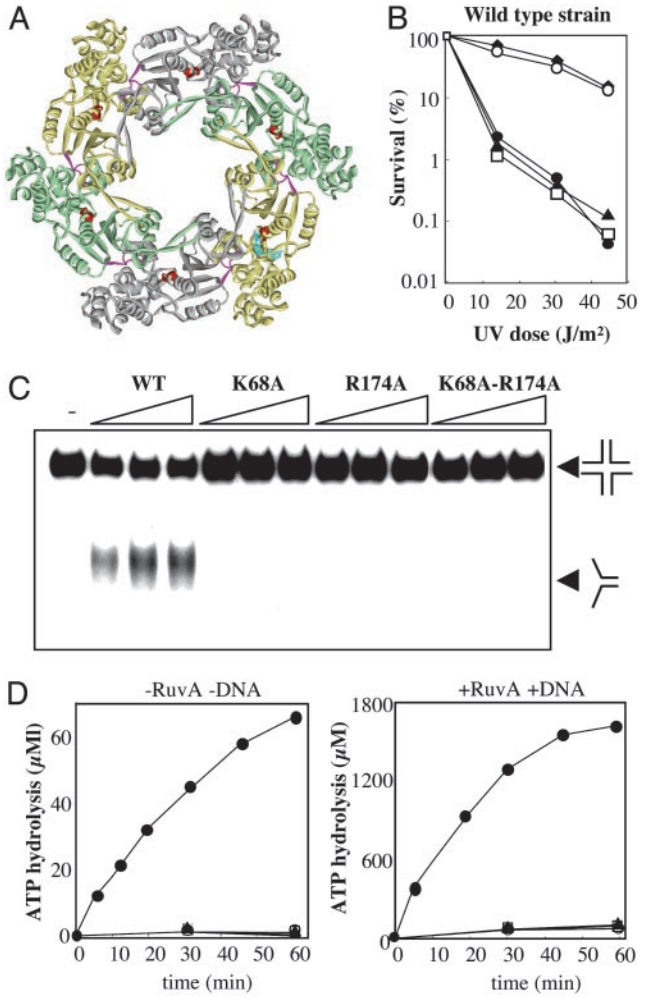

Fig. 1. Characterization of RuvB R174A mutant. (*A*) Arg-174 is positioned proximal to the ATP site of an adjacent molecule. The hexamer model (top view) was constructed by superimposing each ATPase domain of *T. thermophilus* RuvB (AMPPNP form) onto the corresponding region of the simian virus 40 large T antigen crystal structure (36). Each subunit in a hexamer is colored green, gold, or gray. Walker A site and Arg-158 (corresponding to Arg-174 in *E. coli*) are colored red and magenta, respectively. ATP is colored blue. (*B*) UV sensitivity of wild-type strain harboring the mutant *ruvB* genes in a multicopy plasmid. ◆, pRB100 (wild-type RuvB); ○, pAF101(vector); ●, pRB601(K68A); ▲, pRB602(R174A); □, pRB603(K68A–R174A). (*C*) Branch migration activity of mutant RuvB proteins. Reactions were carried out as described in *Materials and Methods*. The concentrations of RuvB proteins were 50, 100, and 200 nM, respectively. (*D*) ATPase activity of mutant RuvB proteins. The ATPase assay was carried out with 1 μM wild-type RuvB (●), K68A (○), R174A (▲), or K68A–R174A (□) in the presence or absence of both DNA and RuvA. The rates of ATP hydrolysis were determined as described in *Materials and Methods*.

wild-type:mutant RuvB ratio was 1:0.8, 1:0.8, or 1:0.3 for K68A, R174A, and K68A–R174A RuvB, respectively (Fig. 2*B*), indicating that R174A and K68A RuvB inhibit wild-type RuvAB-catalyzed branch migration less effectively than the double mutant. Moreover, these inhibition efficiencies suggest that if there is random incorporation of the mutant/wild-type RuvB into the hexamers, then at a 1:0.8 ratio of wild-type/single mutant, the heterohexamers containing one or two mutant RuvB subunits remain active for branch migration. On the other hand, at a 1:0.3 ratio of wild-type/K68A–R174 double mutant, the heterohexamers containing one mutant subunit might be at least competent for the branch migration activity. Therefore, these results imply that RuvB heterohexamers composed of wild-type

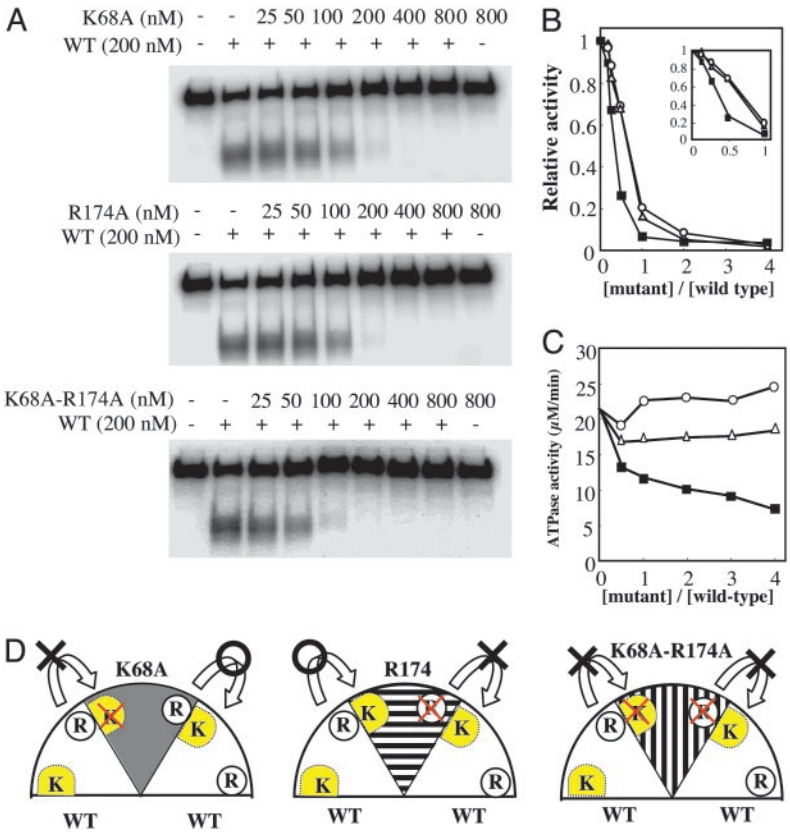

Fig. 2. Branch migration and ATPase activities of the RuvB heterohexamer composed of wild-type and mutant RuvB. (*A*) Branch migration assays were carried out as described in *Materials and Methods*. Reactions were performed with a constant amount of wild-type RuvB and increasing concentrations of RuvB mutants, as indicated. (*B*) Labeled reaction products shown in *A* were quantified by using a phosphorimager. Data obtained at low mutant/wild-type RuvB ratios were expanded in *Inset*. ○, K68A; △, R174A; ■, K68A–R174A. (*C*) ATPase assays were carried out with a constant amount of wild-type RuvB (0.5 μM) and increasing concentrations of mutant RuvB (0.25, 0.5, 1, 1.5, and 2 μM) in the presence of both 0.6 μM RuvA and Holliday junction DNA (100 nM in Holliday junction moles). ○, K68A; △, R174A; ■, K68A–R174A proteins. Results are means of at least three independent measurements. (*D*) Possible roles of Lys-68 and Arg-174 in ATP hydrolysis. Three subunits of the RuvB hexamer are shown. RuvB K68A is defective in ATP hydrolysis but can stimulate the ATPase of the adjacent wild-type RuvB subunit (*Left*). RuvB R174A is defective in stimulating the ATPase of adjacent subunits but can hydrolyze ATP and can be stimulated by Arg-174 of an adjacent wild-type RuvB subunit (*Center*). RuvB K68A–R174A double mutant is defective in both functions (*Right*). The arrows indicate the stimulatory function of Arg-174. The black crosses indicate defective function. ''R'' and ''K'' indicate the R174 and K68, respectively. The yellow box indicates the ATPase site. Each mutation site is indicated by a red cross.

and mutant RuvB might retain some activity for branch migration at Holliday junctions, as was previously observed for heterohexamers composed of wild-type and D113N RuvB (Walker B mutant) (31).

Effect of R174A on ATP Hydrolysis. Previous studies suggest that the defect in RuvB K68A uncouples RuvAB-catalyzed ATP hydrolysis from branch migration (30). Here, a similar pattern is observed for RuvB R174A. Fig. 2*C* shows that RuvB R174A does not inhibit Holliday junction DNA-dependent ATPase of wild-type RuvAB at a 4-fold excess of the RuvB R174A, whereas Fig. 2*B* demonstrates that RuvB R174A does inhibit wild-type RuvAB-catalyzed branch migration. If the homohexamer of RuvB R174A inhibits wild-type RuvB-catalyzed branch migration by competing for binding to the RuvA or Holliday junctions, ATPase activity of wild-type RuvB should be reduced because both RuvA and Holliday junction DNA are required for the elevated ATPase activity of RuvB. However, RuvB R174A does not inhibit the stimulation of wild-type RuvB ATPase activity by RuvA and Holliday junctions. These results strongly support that heterohexamers composed of wild-type and R174A RuvB subunits are formed and present on the RuvA–Holliday junction. One possible explanation for these results is that ATPase-deficient RuvB K68A may undergo an ATP-binding-induced conformational change (30) that allosterically stimulates the ATPase of an adjacent wild-type RuvB through R174 (Fig. 2*D Left*). In contrast, although RuvB R174A is unable to stimulate adjacent wild-type RuvB ATPase, it is competent to hydrolyze ATP in response to the stimulation of the Arg-174 of wild-type RuvB subunit (Fig. 2*D Center*). Because the concentration of wild-type RuvB is kept constant, the total number of catalytically competent ATP sites is not varied in the RuvB heterohexamers by the addition of either K68A or R174A (see Fig. 5, which is published as supporting information on the PNAS web site), which is consistent with the result that the net ATP hydrolysis activity did not change (Fig. 2*C*). In contrast, RuvB K68A–R174A inhibits ATPase by wild-type RuvAB and has a stronger negative effect than single mutant RuvB (Fig. 2*C*), which is consistent with inhibition of wild-type RuvAB-catalyzed branch migration (Fig. 2*A*). RuvB K68A–R174A is incompetent in both stimulating the RuvB ATPase of the adjacent wild-type RuvB and hydrolyzing ATP by itself. Therefore, synergistic dominant negative reduction of wild-type RuvAB ATPase observed for RuvB K68A–R174A may be due to the two distinct defects in ATPase (i.e., catalysis and stimulation of catalysis; Figs. 2*D* and 5). In addition, RuvB K68A–R174A inhibits wild-type RuvAB-catalyzed ATPase activity less effectively than the branch migration activity. These results may reflect the differences in the

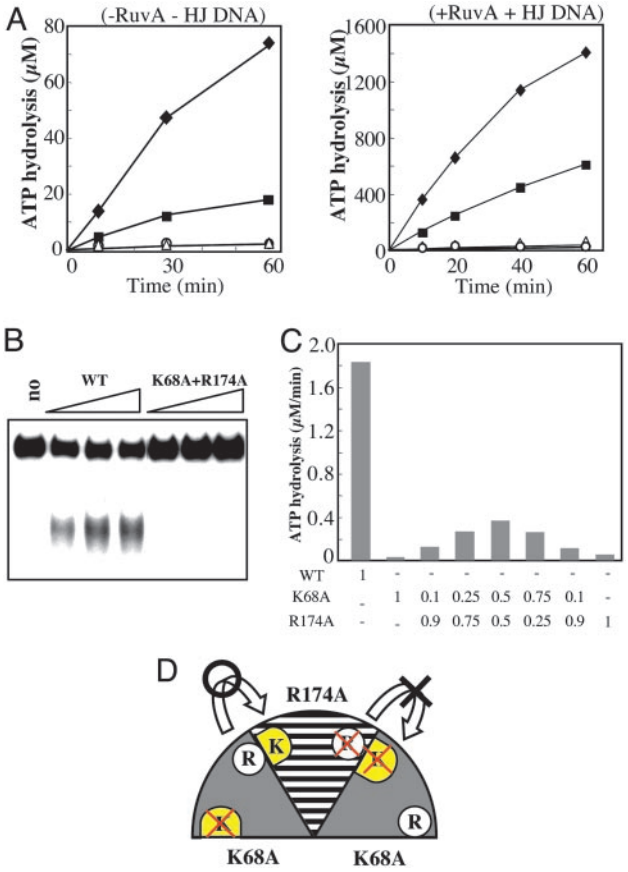

Fig. 3. Restoration of ATP hydrolysis activity by mixing RuvB K68A and RuvB R174A. (*A*) Time course measurements of ATP hydrolysis were carried out with a 1:1 mixture of K68A and R174A RuvB proteins in the absence (*Left*) or presence (*Right*) of both RuvA and Holliday junction DNA. ◆, wild type; ○, K68A; △, R174A; ■, K68A plus R174A proteins. (*B*) Branch migration assays were carried out with increasing concentrations of wild-type RuvB or a 1:1 mixture of K68A and R174A RuvB proteins. The concentrations of RuvB were 50, 100, and 200 nM. Reactions were carried out as described in *Materials and Methods*. (*C*) ATPase activity of mixture containing varying ratios of the K68A and R174A RuvB proteins in the absence of DNA and RuvA. The total RuvB concentration was held constant at 1 μM. (*D*) Possible mechanism of restoration of ATP hydrolysis by mixing K68A and R174A RuvB mutants. Arg-174 of the RuvB K68A subunit interacts with ATP of the RuvB R174A subunit and stimulates ATPase activity of the RuvB R174A subunit. An explanation of the illustration is given in the legend of Fig. 2*D*.

critical numbers of RuvB K68A–R174A subunits required to inhibit ATPase activity coupled or uncoupled with branch migration activity in a heterohexameric RuvB. These data strongly suggest that K68 and R174 in RuvB play catalytic and stimulatory roles in ATP hydrolysis, respectively.

Mixture of K68A and R174A Restores ATPase Activity. The results and interpretation stated above predict that K68A and R174A RuvB will complement each other's deficiency during *in vitro* ATP hydrolysis and branch migration. This possibility was examined by measuring ATPase activity in an equimolar mixture of K68A and R174A RuvB in the presence or absence of RuvA and Holliday junction DNA. The results show partial restoration of RuvB-catalyzed ATP hydrolysis in the presence or absence of RuvA and Holliday junction DNA (Fig. 3*A*). However, branch migration activity was not restored (Fig. 3*B*), suggesting that each active ATPase site within hexamers works independently for the ATP hydrolysis, but cooperative action among the active sites is needed for branch migration. ATPase activity was also

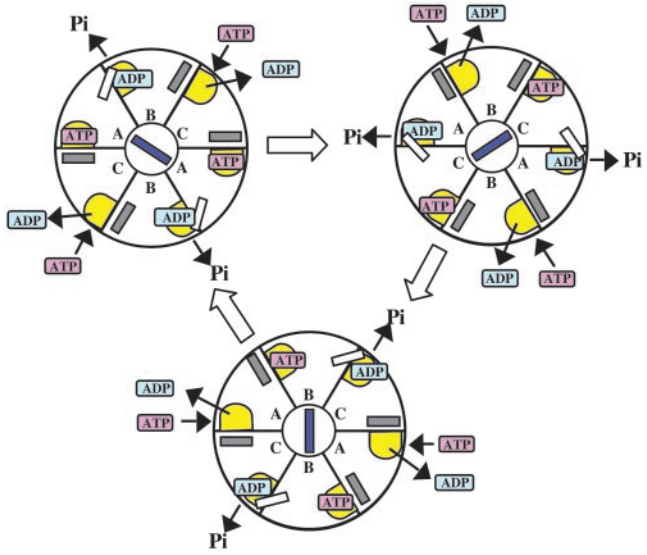

Fig. 4. Model for ATP hydrolysis-coupled branch migration by the RuvB hexamer. Coordinated ATP hydrolysis by the RuvB hexamer is depicted. The paired subunits are lettered A, B, and C. Details are described in the text. Gray and white bars indicate inactive and active forms of Arg-174 residues, respectively. The yellow box indicates the ATPase site. The blue bar indicates double-stranded DNA.

measured with varying ratios of K68A and R174A RuvB with a constant final RuvB concentration of 1 μM. Fig. 3*C* shows that ATPase activity was maximal with an equimolar mixture of the two mutants. Moreover, no ATPase activity was observed in an equimolar mixture of K68A or R174A with K68A–R174A RuvB (data not shown). Thus, under these conditions, RuvB ATPase depends on an intersubunit interaction between K68A and R174A RuvB (Fig. 3*D*), and it is possible that the ATPase defect of K68A and R174A RuvB reflects disruption of this function. As described above, R174 may be critical in this regard as an allosteric effector that plays a critical role during ATP hydrolysis by stimulating the activity of an adjacent subunit in the RuvB hexamer.

ATPase activity was ≈40% of wild-type RuvAB activity when equimolar K68A and R174A RuvB were coincubated with RuvA and Holliday junction DNA. However, based on the assumption that the ATP site of R174A RuvB approached by Arg-174 of the K68A RuvB is catalytically active in K68A/R174A heterohexamers, 25% of the RuvB monomers are expected to be active in a randomly assembled heterohexamer. This result is explained as follows: when wild-type RuvB catalyzes ATP hydrolysis-dependent DNA translocation, RuvB dissociates from the Holliday junction after completion of branch migration. In contrast, K68A/R174A heterohexamers might hydrolyze ATP continuously without promoting branch migration activity (Fig. 3*B*). In other words, the relative high ATPase activity of K68A/R174A heterohexamers may reflect uncoupling of ATP hydrolysis from branch migration. This finding is consistent with the fact that the ATPase of K68A/R174A heterohexamers was 20% of wild-type RuvB ATPase in the absence of RuvA and Holliday junction DNA (Fig. 3*A*).

Mechanism for a RuvB Branch Migration Coupled with the ATP Hydrolytic Cycle. This study presents evidence that intersubunit interaction plays a critical role in ATP hydrolysis by RuvB hexamers and that Arg-174 is essential for this interaction and for sequential hydrolysis reactions. These and other data suggest that the RuvB hexamer includes nonequivalent active sites (i.e., ATP-bound, ADP-bound, and nucleotide-free states) (13, 32–

34), and support a model (Fig. 4) for the hydrolytic cycle of RuvB hexamers. This model proposes that an active RuvB hexamer is composed of one pair each of ATP-bound, ADP-bound, and nucleotide-free monomers. When ATP binds to an ATP-free subunit (subunit A), a conformational change repositions Arg-174 close to the nucleotide phosphates in the adjacent ATP-bound subunit (subunit B) and triggers ATP hydrolysis (Fig. 4 *Upper Left*). Turnover of ADP-bound RuvB is rate-limiting in the hydrolytic cycle. We speculate that ATP-hydrolysis-induced conformational change of the interface involving Arg-174 (subunit B) induces ADP release in subunit C (Fig. 4 *Upper Left*). Indeed, in the RuvB hexamer of *Thermotoga maritime*, Arg-170 (corresponding to Arg-174 in *E. coli*) deviates slightly from the alignment required for ATP hydrolysis when ADP is bound (13). Biochemical data also suggest that ATP binding induces ADP release in other binding sites (33). Consequently, we propose that ADP release (subunit C) and ATP hydrolysis (subunit B) are regulated by the binding of ATP to subunit A (Fig. 4 *Upper Left*). In other words, ATP is an allosteric effector of ATP hydrolysis and ADP release. One benefit of this mechanism is that it may prevent ATP hydrolytic cycles when RuvB does not form a hexamer.

Domain III of RuvB is a putative DNA binding domain that resembles a winged-helix domain. A RuvB mutant with an R318C mutation in the winged-helix domain is defective in *in vivo* DNA repair and plays an important role in DNA binding *in vitro* (15). In structural models of *T. thermophilus* and *T. maritima* RuvB, it is likely that domain III binds DNA when ATP is bound (13, 34). Moreover, another structural model based on the hexameric replication factor C small subunit of *Pyrococcus furiosus* (RFCS) also suggests that domain III is located close to the central hole accommodating double-stranded DNA (35). Therefore, it is possible that step-wise rotation through the RuvB hexamer from ATP-bound to ADP-bound to nucleotide-free states might couple ATP hydrolysis and DNA translocation (Fig. 4). Further studies are needed to determine how nucleotide binding or hydrolysis (and associated conformational change in the RuvB hexamer) drives DNA translocation with respect to the RuvAB complex. However, it is clear that conserved R174 plays a key role in coordinated ATP hydrolysis by *E. coli* RuvB.

Previous studies established a similar mechanism for arginine fingers of GTPase-activating proteins bound to GTP binding proteins such as Ras or Rho (16–20). The mechanistic insights revealed by this study may apply to oligomeric AAA/AAA$^+$ proteins and GTPase-activating protein/GTP-binding protein complexes and facilitate future efforts to exploit and/or manipulate the biological functions of these important enzymes, which play diverse roles in critical biological processes such as proteolysis, protein stability and folding, organelle formation, cell cycle progression, and DNA repair and replication.

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