FORMATION OF A STABLE RuvA DOUBLE TETRAMER IS REQUIRED FOR EFFICIENT BRANCH MIGRATION IN VITRO AND FOR REPLICATION FORK REVERSAL IN VIVO

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Running title: Crucial role for RuvA octamers in replication fork reversal

In bacteria, RuvABC is required for the resolution of Holliday junctions (HJ) made during homologous recombination. The RuvAB complex catalyzes HJ branch migration and replication fork reversal (RFR). During RFR, a stalled fork is reversed to form a HJ adjacent to a DNA double-strand end, a reaction that requires RuvAB in certain E. coli replication mutants. The exact structure of active RuvAB complexes remains elusive as it is still unknown whether one or two tetramers of RuvA support RuvB during branch migration and during RFR. We designed an E. coli RuvA mutant, RuvA2 KaP, specifically impaired for RuvA tetramer-tetramer interactions. As expected, the mutant protein is impaired for complex II (two tetramers) formation on HJs, although the binding efficiency of complex I (a single tetramer) is as wild-type. We show that although RuvA complex II formation is required for efficient HJ branch migration in vitro, RuvA2 KaP is fully active for homologous recombination in vivo. RuvA2 KaP is also deficient at forming complex II on synthetic replication forks and the binding affinity of RuvA2 KaP for forks is decreased compared to wild type. Accordingly, RuvA2 KaP is inefficient at processing forks in vitro and in vivo. These data indicate that RuvA2 KaP is a separation-of-function mutant, capable of homologous recombination but impaired for RFR. RuvA2 KaP is defective for stimulation of RuvB activity and stability of HJ-RuvA-RuvB tripartite complexes. This work demonstrates that the need for RuvA tetramer-tetramer interactions for full RuvAB activity in vitro causes specifically a RFR defect in vivo.

The RuvAB complex is a highly sophisticated molecular machine, which carries out branch migration of Holliday junctions during homologous recombination (HR). RuvA binds specifically to four-armed Holliday junctions (HJ) and guides the assembly of two RuvB hexameric rings onto diametrically opposite arms of the HJ. RuvB, a AAA+ ATPase (1), is the motor which drives branch migration of the crossover point (1-3). After branch migration, a dimer of RuvC resolves the HJ by making two sequence-specific symmetrical cuts, producing either patched or spliced linear products (4-6). Genetic studies showed that RuvC cannot function in vivo in the absence of RuvAB (7,8) and it has been proposed that a RuvABC complex, known as the resolvasome, allows RuvC to scan for cleavable sequences (3,9-11). RuvA binds to HJs in vitro as one tetramer (complex I) or two tetramers that sandwich the junction (complex II), in a concentration-dependent manner; but it
is not clear whether the RuvAB complex contains one or two tetramers of RuvA in vivo (12-21). A RuvAB branch migration complex made of two RuvA tetramers would prevent access of RuvC to the Holliday junction. Whether to form the resolvosome the RuvC dimer displaces one of the two RuvA tetramers present in the RuvAB complex, or whether the RuvC dimer simply binds opposite a single RuvA tetramer present in the complex is a currently unanswered question.

In addition to processing Holliday junctions in homologous recombination, RuvAB plays an important role upon DNA replication inactivation. In certain E. coli replication mutants, stalled replication forks undergo a process known as replication fork reversal (RFR) (22). As the stalled fork is reversed, the newly synthesised strands are unwound from the daughter duplexes and base-pair to form a Holliday junction, known as a reversed fork. Branch migration of the reversed fork by RuvAB drives extrusion of an arm with a duplex end which allows entry of RecBCD. RecBCD can either reset the fork by degrading the duplex, or load RecA, which carries out invasion and strand exchange with the homologous duplex at the replication fork, to reset a new fork. Both pathways result in PriA-dependent replication restart (23). Intriguingly, RuvAB are actually required for replication fork reversal to occur in dnaEts, holD Q10am mutants (24). Yet in vivo, RuvAB preferentially unwinds synthetic replication forks in a direction that is opposite the direction for fork reversal (25). RuvAB could reverse model replication forks in vitro if RuvB was only allowed to form one hexameric ring on the parental duplex of the fork (25). It has been speculated that in vivo, asymmetric binding of a single RuvA tetramer onto a three-armed fork may result in asymmetric loading of a single RuvB hexamer onto the parental duplex (24). Alternatively, cellular factors may force RuvA to load RuvB in this manner.

In a RuvA octamer, the two tetramers do not only interact with DNA but also with each other through four contacts involving domain II of each monomer. Specifically, six ionic interactions form between the α-helix 6 in domain II of each opposite monomer resulting in four points of contact between tetramers (18) (supplementary Fig. 1). The role of RuvA octamers for efficient branch migration has been investigated using RuvA mutants designed to disrupt the tetramer-tetramer interface and prevent complex II formation. A triple E. coli RuvA mutant, RuvA3m, was unable to form complex II at RuvA concentrations of up to 2 μM and was deficient in processing synthetic HJs in vitro and in vivo (26). Unexpectedly, RuvA3m helicase activity and branch migration of Y-junctions in vitro appeared unaffected and it was proposed that complex I was able to support one RuvB hexameric ring but complex II was needed to assemble two hexameric rings on the Holliday junction (26). A Thermus thermophilus “tetramer only” RuvA mutant, RuvA(DK) (L125D and E126K) was also studied. Electron microscopy demonstrated that a single RuvA(DK) tetramer formed tripartite complexes containing two hexameric rings of RuvB on the HJ (27). RuvA(DK) displayed reduced ability to promote branch migration of Holliday junctions in vitro and, significantly, could not support branch migration with a single RuvB hexamer (28). The capacity for replication fork reversal of these “tetramer-only” mutants has not been tested.

Recently, two ruvA mutants called ruvAZ3 and ruvAZ38 (H29R K129E F140S and N79D N100D, respectively) were isolated and characterised as separation-of-function mutants that can process Holliday junctions but cannot reverse replication forks (29). The RuvAz proteins contain several mutations in different domains of the proteins, making it difficult to ascertain the molecular cause for their phenotypes. One intriguing observation is the inability of RuvAz mutants to form complex II on Holliday junctions, which is common with the RuvA3m and RuvA(DK) mutants discussed above. However, the tetramer-only RuvAz mutants are fully capable of promoting homologous recombination in vivo, whereas the tetramer-only RuvA3m is not.

In order to understand how the ability of RuvA to form octamers (complex II) relates to branch migration of HJs and replication fork reversal in vitro and in vivo, we have designed a new “tetramer-only” RuvA mutant, RuvA2 KaP. RuvA3m, which was used in previous work (26), is inactive in vivo and displays significant non-specific DNA binding which could explain some discrepancies compared with studies of RuvA(DK) (28). In contrast, RuvA2 KaP was designed to only disrupt tetramer-tetramer interactions. In this study we have investigated the ability of RuvA2 KaP to bind and process HJs and synthetic forks in vitro and how these
correlate with the ability of the mutant to process different substrates in vivo. We also compare certain biochemical activities of RuvA2kap mutant with those of the separation-of-function mutants RuvA3 and RuvA87.

EXPERIMENTAL PROCEDURES

Mutagenesis of RuvA2kap — In order to test RuvA2kap function in vivo, plasmids pGB-ruvA and pGB-ruvAB were mutagenised using mutagenic primers 259F and 260R (Supplementary Table 2). Both of these primers incorporated a codon change from GAA to CGC at position 379 in the coding sequence. Site-directed mutagenesis (QuikChange® II Site-Directed Mutagenesis Kit) was used to produce pGB-ruvA1kap and pGB-ruvA1kapB which contained a single amino acid substitution: E127R. The thermal cycling procedure employed was as follows: 1 X 95°C 30 s, 16 cycles of 95°C for 30 s, 53°C for 45 seconds and 68°C for 10 minutes. In order to produce pGB-ruvA2kap and pGB-ruvA2kapB, which contain two amino acid substitutions E127R and K119A, pGB-ruvA1kap and pGB-ruvA1kapB were used as templates and the primers 401F and 402R were used to introduce the second amino acid substitution K119A. Both the primers 401F and 402R incorporated a codon change from AAA to GCA at position 355 in the coding sequence. The mutagenesis was carried out as described to introduce the first substitution (E127R). Sequencing of the pGB-ruvA1kap, pGB-ruvA2kap, pGB-ruvA1kapB and pGB-ruvA2kapB constructs was carried out by MWG using the primers RkF, RkR and internal primers RkiF and RkiR (Supplementary Table 2).

Recombinant Protein production — All protein fractions were diluted with loading buffer (0.125 M Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 0.01 % bromophenol blue, 10 % β-mercaptoethanol) and analyzed by SDS PAGE analysis. RuvA or RuvA2kap was cloned into pET21a and expressed in BL21-GOLD(DE3) and purified following a protocol described in (26) with several modifications. For each protein, four frozen pellets (total volume 4 ml) were thawed on ice and re-suspended in 21 ml of Lysis buffer (100 mM Tris-HCl pH 8, 5 % glycerol, 2 mM EDTA, 1 mM of DTT and 1 mg.ml⁻¹ of lysozyme) and incubated for 30 minutes at + 4°C. A final concentration of 1 M NaCl and 0.1 % Triton X-100 was added to the solution and incubation continued for a further 10 minutes on ice. The solution was made up to 0.4 % Sodium deoxycholate and spun at 42 k.r.p.m. for 60 minutes at + 4°C in a Type 70 Ti rotor in an Optima™ L-100 XP ultracentrifuge (Beckman Coulter). The supernatant was dialysed against 2 litres of TEGD buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM DTT, 10 % glycerol).

The crude lysate was loaded onto a DEAE column and the column was washed with TEGD buffer and a gradient of 0 mM – 500 mM KCl in TEGD buffer was employed to elute the protein. The eluted protein was dialysed against 2 litres of H buffer (10 mM KPi pH 6.8, 150 mM KCl, 0.5 mM DTT, 10 % glycerol) at +4°C. Dialyzed protein was loaded onto a Hydroxylapatite column and the column was washed with H buffer. A 100 ml 10 mM – 600 mM gradient of KPi in H buffer was used to develop the column. Eluted RuvA was dialysed at + 4°C against 2 litres of H buffer supplemented with 5 mM β-mercaptoethanol, 0.1 mM PMSF and 100 mM KCl with no DTT. Dialyzed RuvA was loaded onto a HiTrap Heparin column which was washed with buffer and a 10 mM – 600 mM KPi gradient was used to elute the protein which was dialysed against 2 litres of TEGD buffer at + 4°C. RuvB was loaded onto an ssDNA column and the column was washed with TEGD buffer and developed with a 0 mM – 1M KCl gradient. Eluted RuvA protein was dialysed overnight at + 4°C against 2 litres of TEGD buffer. Dialysed RuvA protein was loaded onto a 1 ml MonoQ column which was washed with TEGD buffer and a gradient 0 M – 1.5 M KCl in TEGD buffer was used to elute the RuvA protein. The protein concentrations of the purified RuvA protein were determined using a Bradford assay. RuvA was stored as either 10 % or 50 % glycerol stocks at – 20°C. RuvA2kap protein was produced using an identical procedure except after purification on a Heparin HiTrap column the protein was loaded straight onto a MonoQ column; the ssDNA column was not used.

A protocol for producing RuvBD113E was modified to produce wild-type RuvB (26). RuvB was over-expressed from plasmid pET21a in BL21-GOLD(DE3) cells. Cultures of RuvB-pET21a were grown in LB (100 μg.ml⁻¹ ampicillin) at 37°C to an O.D. of 0.6 at 600 nm. The cultures were supplemented with 1 mM IPTG and incubated for six hours at 37°C at 250 RPM. The cultures were pelleted at 4000 RCF
for 10 minutes in a SLA 3000 rotor in a Sorvall RC6 Plus centrifuge and resuspended in 25 ml of LB. The suspension was further spun for 10 min at 4000 RCF at + 4°C and the resulting pellets frozen at − 20°C. Purification was carried out as in (26).

RuvC was expressed and purified using a protocol described in (5).

**Size Exclusion Chromatography (SEC)** — RuvA, RuvA mutants and RuvB proteins were dialysed against TEGD supplemented with 0.1 M NaCl overnight at + 4°C. A total volume of 200 μl of 250 μg of each protein was applied to the 25 ml Superose 6 TM 10/30 GL column. The proteins were eluted from the column in TEGD buffer supplemented with 0.1 M NaCl at a flow rate of 0.3 ml.min⁻¹. Molecular weights of species were estimated by comparison with 5 molecular weight standards (BioRad). Fractions were analysed by SDS PAGE analysis and the UV absorbance profiles of the eluted proteins were recorded.

**DNA substrate preparation** — The DNA substrate X12 (HJ) was constructed as described (26) see Supplementary Table 1. Replication fork-like substrates: F2 and HJY3m, were also constructed. JBM5a and IT01 were synthesised with a fluoro-tag IRD700 attached to the 5’ end. The required combinations of oligonucleotides were included in annealing reactions, using 1 μg of each oligo in SSC buffer (150 mM sodium chloride and 15 mM trisodium citrate, pH 7.0) incubated at 95°C for 2 minutes and slowly annealed by cooling the heat block to room temperature. Substrates were purified by electrophoresis on an 8 % native gel in TBE buffer run at 15 V.cm⁻¹ for 80 minutes at + 4°C. Gel bands were cut from the gel and DNA was eluted from the gel pieces by electrophoresis in 0.5 X TBE buffer for 1 hour using a BioTrap Multikit (Schleicher and Schuell). Each DNA substrate was then dialysed against 2 litres of DNA storage buffer (10 mM Tris-HCl pH8, 1 mM EDTA, 50 mM NaCl) and stored at − 20°C.

**EMSA** — EMSA reactions containing the indicated amount of protein and DNA substrate were incubated in DNA binding buffer (50 mM Tris-HCl pH 7.5, 15 mM MgCl₂, 2 mM ATP, 2 mM DTT and 100 μg.ml⁻¹ BSA). Proteins were diluted using Dilution buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.5 mM DTT, 10 % glycerol). Reactions were incubated for 5 minutes on ice. For gel loading, 5 μL of 80 % glycerol was added to each 20 μL reaction to allow the samples to sink into the wells; no dye was used as the dye interferes with detection of the signal. In some instances protein-DNA complexes were resolved on native gels of different concentrations of polyacrylamide: between 4 % and 10 % in TBE buffer or in low ionic TAE buffer (6.7 mM Tris-HCl pH 8.1, 2 mM EDTA, 3.2 mM sodium acetate) at 6 V.cm⁻¹ at + 4°C in a Bio-Rad miniprotein II gel system. The gels were run at 10 V.cm⁻¹ for 4 hours at + 4°C. Gels were scanned using the Odyssey ® Infrared Imaging System (from LI-COR Biosciences) at 700 nm at an intensity of 10.

For EMSAs in Mg²⁺, reactions were carried out exactly as for DNA binding assays in EDTA, except EDTA was omitted from the DNA Binding buffer and replaced by 5 mM MgCl₂. The samples were loaded after addition of 5 μl 80 % glycerol and resolved using 6 % polyacrylamide native gels with the EDTA omitted from the gel and replaced with 5 mM MgCl₂. The gels were run in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 200 μM MgCl₂) at 6 V.cm⁻¹ for 4 hours with recirculation of the buffer on ice.

**Branch migration assays** — Reactions were carried out in branch migration buffer (20 mM Tris-HCl pH 7.5, 15 mM MgCl₂, 2 mM ATP, 2 mM DTT and 100 μg.ml⁻¹ BSA). DNA and then protein were added to the reactions at the required concentrations. The reaction was incubated at 37°C for 30 min then 5 μl of 5 X Stop buffer (100 mM Tris-HCl pH 8, 200 mM EDTA, 2.5 % SDS and 10 mg.ml⁻¹ Proteinase K) was added to each 20 μl reaction with incubation at room temperature for 10 minutes. 5 μl of 80 % glycerol was added to each sample and the samples were loaded onto 6 % polyacrylamide native gels and run in 0.5 X TBE buffer at 7 V.cm⁻¹ for 2 hours. DNA products were visualized on the Odyssey® Infrared Imaging System at 700 nm at intensity 10.

**RuvA/B-DNA complex formation assay** — Reactions were incubated at + 37°C for 30 minutes in formation buffer (20 mM triethanolamine-HCl pH 7.5, 10 mM MgCl₂, 0.25 mM ATPγS, 1 mM DTT, 50 μg.ml⁻¹ BSA). The proteins and DNA were added as required and were fixed by adding 0.2 % glutaraldehyde and incubating reactions for 20 minutes at 37°C. 5 μl of 80 % glycerol were added to each reaction and samples were loaded onto a 6 % polyacrylamide native gels and run in 1X TAE buffer. The reaction was performed in TBE buffer (45 mM Tris base, 45 mM boric acid, 200 μM MgCl₂) at 6 V.cm⁻¹ for 4 hours with recirculation of the buffer on ice.

**Size Exclusion Chromatography (SEC)** — RuvA, RuvA mutants and RuvB proteins were dialysed against TEGD supplemented with 0.1 M NaCl overnight at + 4°C. A total volume of 200 μl of 250 μg of each protein was applied to the 25 ml Superose 6 TM 10/30 GL column. The proteins were eluted from the column in TEGD buffer supplemented with 0.1 M NaCl at a flow rate of 0.3 ml.min⁻¹. Molecular weights of species were estimated by comparison with 5 molecular weight standards (BioRad). Fractions were analysed by SDS PAGE analysis and the UV absorbance profiles of the eluted proteins were recorded.

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buffer. Gels were run at 10 V.cm⁻¹ for 2 hours at room temperature and scanned on the LI-COR fluoro imaging system at 700 nm intensity 10.

**RuvC cleavage assay** — Reactions were carried out in cleavage buffer (50 mM Tris-HCl pH 8, 10 mM MgCl₂, 50 mM KCl, 5 mM β-mercaptoethanol, 100 μg.ml⁻¹ BSA and 5 % glycerol). 2 μl of RuvA protein and 2 μl of RuvC protein were mixed and incubated with HJ in cleavage buffer at + 37°C for one hour. 5 μl of 5x stop buffer was then added to the 20 μl reactions which were incubated at + 37°C for a further 10 minutes. 5 μl of 80 % glycerol was added to 25 μl reactions which were loaded onto 6 % native PAGE gels and run in TBE buffer at 10 V.cm⁻¹ at room temperature. DNA products were visualized using the Odyssey LI-COR fluoro imaging system.

**ATPase assay** — The indicated amounts of protein and DNA substrate were mixed and reactions were performed in ATPase buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 15 mM MgCl₂, 0.1 mg.ml⁻¹ BSA, 1 mM DTT, 15 mM MgCl₂ and 0.5 mM ATP). A Malachite green kit was used to detect activity, the reactions were pre-incubated on ice and a zero time point was taken with 20 μl of reaction added to 5 μl of 0.5M EDTA. Reactions were then incubated at 37ºC with time points taken at 5 min, 15 min and 30 min. 150 μl of ALS mix (Innova Biosciences) was added to each well and the reactions were incubated for 30 min at room temperature. The 96 well plates were scanned in a Tecan Sunrise fluorometer and analysed with software by Magellan. A calibration curve of 10 different KPi concentrations was used to determine the amount of phosphate released in each reaction.

**Measurement of recombinational DNA repair** — UV irradiation was performed as described (29). The survival ratio was a comparison of colonies that grew on a replica control plate compared to colonies that grew on a plate which was irradiated. For mitomycin C treatment, cells were grown at 37°C in LB to an OD₆₀₀ = 0.5, mitomycin C was added to the culture at a final concentration of 2 μg/ml and incubation continued at 37°C for 90 minutes. An untreated culture was used as control. Appropriate dilutions were plated on LB plates and incubated over-night at 37°C. Ratios of cfu of mitomycin C treated: cfu of untreated cells were calculated.

**Measurement of conjugational recombination** — Conjugations were performed as described using JJC145 as Hfr donor (29); donor and recipient cells were mixed for 25 min. Selective medium was M9 minimal medium supplemented with leucine, proline, threonine and arginine (2% final concentration each) and 10 μg.ml⁻¹ chloramphenicol.

**Measure of Linear DNA by PFGE** — Quantiﬁcation of pulsed ﬁeld gels was performed using in vivo ³H-thymidine labelled chromosomes as previously described (22).

**RESULTS**

**RuvA2KaP binds efficiently to Holliday Junctions as a single tetramer.**

A set of RuvA mutants were generated by introducing amino acid substitutions in α-helix-6 in domain II, which is the region involved in tetramer-tetramer interactions. Specifically, the RuvA2KaP used in this study carried E127R and K119A mutations in the tetramer-tetramer interface (supplementary Fig. 1). The overall electrostatic charge of this region changes only slightly compared to wild-type and the slightly more basic/positive charge does not alter RuvA2KaP interaction with DNA (Fig 1A). The mutant RuvA3m used in a previous study formed aberrant complexes on HJs, probably caused by the significant increase in positive charge in α-helix-6 (26).

The ability of RuvA2KaP to form stable complexes was tested in vitro, RuvA2KaP was analysed on SDS PAGE gels and formed tetramers comparable to wild-type RuvA, which is stable enough not to dissociate in SDS (supplementary Fig. 2A). When boiled and loaded onto an SDS PAGE gel, RuvA2KaP dissociated into monomers, indicating that, like RuvA, the mutant forms stable tetramers that only dissociate upon boiling (supplementary Fig. 2A). RuvA2KaP was analysed by size exclusion chromatography (SEC) on a 25 ml Superose 6 TM 10/30 GL column and the elution profile of RuvA2KaP was comparable to RuvA (supplementary Fig. 2B). Additionally, RuvA2KaP mixed with RuvB was loaded onto a 25 ml Superose 6 TM 10/30 GL column and the formation of RuvA2KaPB complex was equivalent to RuvAB complex formation (supplementary Fig. 2C). In conclusion, RuvA2KaP forms a stable tetramer and is able to form a complex with RuvB in solution with an efficiency comparable to that of wild-type RuvA.
The binding of $\text{RuvA}_{2\text{KaP}}$ to a fluoro-tagged synthetic HJ (X12) was tested using EMSAs (Fig. 1A). In EDTA-containing buffer, RuvA formed both complex I and II at lower concentrations, but exclusively formed complex II at protein concentrations of 150 nM and higher. Conversely, $\text{RuvA}_{2\text{KaP}}$ only formed complex I, even at protein concentrations as high as 2 $\mu$M. The amount of HJ bound by $\text{RuvA}_{2\text{KaP}}$ was similar to wild type RuvA (Fig. 1B), indicating that the mutations did not affect the affinity of $\text{RuvA}_{2\text{KaP}}$ for HJs. Holliday junction binding was further tested in the presence of Mg$^{2+}$ as RuvB requires at least 5 mM of Mg$^{2+}$ for efficient ATP hydrolysis and branch migration. In 5 mM MgCl$_2$, wild type RuvA bound to X12 exclusively as complex II, even at low protein concentrations, leaving a significant amount of free junction (Fig. 1C). $\text{RuvA}_{2\text{KaP}}$ formed complex I at concentrations up to 75 nM, but at 250 nM of $\text{RuvA}_{2\text{KaP}}$ and above, complex II also formed (Fig. 1C). The overall $\text{RuvA}_{2\text{KaP}}$ binding of X12 in Mg$^{2+}$ was comparable with RuvA (Fig. 1D). It was clear that $\text{RuvA}_{2\text{KaP}}$ complex II was stabilised by Mg$^{2+}$ so we checked for a direct stabilising effect of 5 mM MgCl$_2$ on tetramer-tetramer interactions using SEC. As the SEC elution profile of RuvA remained unchanged (data not shown), the effect of Mg$^{2+}$ was therefore dependent on the presence of DNA. Experiments in Mg$^{2+}$ buffer reveal that a lack of tetramer-tetramer interaction does not fully prevent complex II formation but renders it dependent on a high concentration of RuvA protein.

Stability of $\text{RuvA}_{2\text{KaP}}$ complex II in solution — In vivo RuvC cannot function without RuvAB (7,8,29,30); however, it has been shown that formation of RuvA complex II occludes the HJ and prevents RuvC-mediated cleavage in vitro (26,31,32). To assess the stability of the $\text{RuvA}_{2\text{KaP}}$ complex II in the presence of Mg$^{2+}$, we compared the ability of both wild type and mutant proteins to protect a HJ from cleavage by RuvC (Fig. 2). Varying concentrations of RuvA or $\text{RuvA}_{2\text{KaP}}$ were incubated with X12, followed by addition of 100 nM RuvC. Significant inhibition of RuvC HJ cleavage was observed at wild type RuvA concentrations of 100 nM and cleavage was completely abolished at 300 nM RuvA. The inhibition of RuvC cleavage correlated well with complex II formation in EDTA binding studies but only roughly with the concentration at which complex II forms in the Mg$^{2+}$binding studies. HJ cleavage by RuvC was also tested in the presence of up to 2 $\mu$M of $\text{RuvA}_{2\text{KaP}}$. Cleavage was only slightly inhibited within the range of 100 – 500 nM $\text{RuvA}_{2\text{KaP}}$ and was still weakly observed in the presence of 2 $\mu$M of $\text{RuvA}_{2\text{KaP}}$ (Fig. 2A). This demonstrates that although $\text{RuvA}_{2\text{KaP}}$ forms complex II on Holliday junctions, the stability of $\text{RuvA}_{2\text{KaP}}$ complex II is reduced compared to RuvA complex II.

Because the $\text{RuvA}_{2\text{KaP}}$ protein confers to E. coli a separation-of-function phenotype (see below), we compared the previously isolated separation-of-function mutants RuvAz3 and RuvAz87 to $\text{RuvA}_{2\text{KaP}}$ using the same conditions. As with $\text{RuvA}_{2\text{KaP}}$, both RuvAz mutants were unable to inhibit RuvC cleavage of the junction at concentrations up to 300 nM (Fig. 2B). The inability of RuvAz mutants to protect the HJ from RuvC cleavage confirms that they form unstable complex II (29). These results also suggest that a tetramer of RuvA bound to the HJ (complex I) does not inhibit HJ cleavage by RuvC and it is possible that both RuvA and RuvC bind together to the junction. Alternatively, complex I might transiently dissociate from the junction allowing RuvC access, whereas complex II does not dissociate.

Stimulation of RuvB ATPase activity by RuvA mutants — To measure the functional interaction between $\text{RuvA}_{2\text{KaP}}$ and RuvB, we tested the ability of $\text{RuvA}_{2\text{KaP}}$ to stimulate the DNA-dependent ATPase activity of RuvB. Time courses of ATP hydrolysis using 100 nM of RuvA or $\text{RuvA}_{2\text{KaP}}$, 500 nM RuvB and 5 ng X12 are shown in Fig. 3A. The DNA-dependent ATPase activity of $\text{RuvA}_{2\text{KaP}}$ was about half of the activity of wild type RuvAB: RuvAB hydrolysed 80 $\mu$mol of ATP in 30 minutes while $\text{RuvA}_{2\text{KaP}}$ hydrolysed 40 $\mu$mol of ATP in 30 minutes (Fig. 3A). The ATPase activity was also measured with higher RuvA concentrations. ATP hydrolysis was 2 to 3-fold lower with $\text{RuvA}_{2\text{KaP}}$ compared to RuvA at all concentrations at which $\text{RuvA}_{2\text{KaP}}$ complex II formation was observed by EMSA (250 nM and above) (Fig. 3B). These data indicate that tetramer-tetramer interactions within complex II are necessary for optimal stimulation of the DNA-dependent ATPase activity of RuvB.

The ability of $\text{RuvA}_{2\text{KaP}}$ mutant to stimulate the DNA-dependent ATPase activity of RuvB was compared with that of the separation of function mutants RuvAz3 and RuvAz87. Fig. 3C shows the amount of ATP hydrolysed per
mol of RuvB per min in the presence of ΦX174 virion DNA for different RuvA mutants. RuvB alone hydrolysed about 8 mol of ATP per mol of RuvB.min⁻¹. The presence of RuvA stimulated RuvB to hydrolyse 50 mol of ATP per mol of RuvB.min⁻¹. RuvA2Kap, RuvAz3 and RuvAz87 stimulated RuvB to hydrolyse 18, 20 and 16 mol of ATP per mol of RuvB.min⁻¹ respectively. In the absence of DNA, neither of RuvA, RuvA2Kap, RuvAz3 or RuvAz87 stimulated the hydrolysis of ATP by RuvB, as expected (data not shown). These data show that the three mutants RuvA2Kap, RuvAz3 and RuvAz87 are less efficient in stimulating RuvB ATPase activity than wild type RuvA.

RuvA2Kap forms unstable RuvA-RuvB-HJ tripartite complexes in solution—The RuvA2Kap mutant was found to bind RuvB in solution by SEC analysis as wild-type RuvA (supplementary Fig. 2B and 2C). To analyse the interactions of RuvA2Kap with RuvB on Holliday junctions (X12), these large protein-DNA complexes were cross-linked with 0.25 % glutaraldehyde and analysed by native PAGE. When incubated with 5 ng of X12, 400 nM of RuvA alone could be cross-linked on DNA as complex II but 400 nM of RuvA2Kap could not (Fig. 4 lanes 10 and 11). The inefficient glutaraldehyde cross-linking of RuvA2Kap complex II on X12 reveals the reduced stability of the RuvA2Kap tetramer-tetramer interaction across the junction. Increasing concentrations of RuvA or RuvA2Kap were incubated with 300 nM RuvB and X12. RuvB alone could not be cross-linked to X12 under these conditions (Fig. 4 lane 12). RuvAB complexes on X12 are detected when 25 nM RuvA and above is used (Fig. 4 lanes 1 and 2). At 200, 300 and 400 nM RuvA, complex II is also observed in addition to RuvAB complexes on DNA (Fig. 4 lanes 4, 5 and 6). The stoechiometry of RuvA:RuvB on the junction is predicted to be 8:12 monomers respectively, thus at equal concentrations of RuvA and RuvB there is an excess of RuvA which forms complex II. We incubated 300 and 400 nM RuvA with 600 nM RuvB (Fig. 4, lanes 7 and 8). This altered the ratio of RuvA:RuvB to favour formation of RuvAB complexes on DNA rather than complex II (Fig. 4, lanes 7 and 8). Interestingly, only a fraction of X12 could be cross-linked with the mutant RuvA2KapB compared to wild type RuvAB and a significant amount of DNA remained protein-free (Fig. 4 lanes 13 to 18). Increasing the concentration of RuvB to 600 nM had no effect on tripartite RuvA2Kap-RuvB-HJ complex formation (Fig. 4 compare lanes 17 and 18 with 19 and 20). The cross-linking experiments demonstrate the reduced stability of RuvA2Kap complexes with RuvB on DNA. Thus the stability of the RuvA double tetramer is crucial for the formation of a stable tripartite RuvAB-HJ complex.

RuvA2Kap–RuvB mediated branch migration of synthetic Holliday junctions—The ability of RuvA2Kap and RuvB to promote branch migration of HJs was tested using fluoro-tagged X12, increasing concentrations of RuvA or RuvA2Kap and 250 nM of RuvB. As shown in Fig. 5A, X12 was processed efficiently by wild type RuvAB to form two branch migration products. In contrast, RuvA2Kap was clearly deficient in branch migration with small amounts of products observed at RuvA2Kap concentrations of 75 nM and above. Around 75 % of X12 was processed at 100 nM wild type RuvA, while less than 25 % of X12 was dissociated at 100 nM RuvA2Kap (Fig. 5B). We conclude that branch migration was inefficient due to the weak stability of RuvA2Kap complex II.

Holliday junction branch migration with a single RuvB hexamer was tested using the synthetic junction HJY3-hm (28). HJY3-hm is made of three short 14-bp arms and one 49-bp arm, so that only one RuvB hexamer can load onto the long arm of this junction. As with X12, the binding affinities of wild type RuvA and RuvA2Kap to HJY3-hm were comparable (data not shown). The assays with HJY3-hm showed highly efficient RuvAB-promoted branch migration of the junction through its short arms but RuvA2Kap complex was inefficient (Fig. 5C). At 25 nM RuvA, HJY3-hm dissociated completely, but in contrast, only 25 % of the substrate was processed by 25 nM RuvA2Kap (Fig. 5C). At 200 nM RuvA2Kap, only 35 % of the HJY3-hm was processed (Fig. 5C). These results show that a single RuvB hexamer assembled on DNA requires stable interactions between two RuvA tetramers for efficient processing of a four-way junction.

Binding and processing of synthetic Replication Forks by RuvA2Kap—We tested the ability of RuvA2Kap to bind and process a synthetic model replication fork (F2), in which the three branches of the forks are fully double stranded. In EDTA buffer, RuvA bound to F2 exclusively as complex II at concentrations above 10 nM but RuvA2Kap only formed complex I at all concentrations tested (Fig. 6A).
Significantly, RuvA2_Kap showed reduced affinity of binding to forks compared with RuvA (Fig. 6B) even though the binding affinity of RuvA2_Kap to HJs was comparable to that of RuvA. Similar results were obtained in the presence of Mg^{2+} - above 10 nM RuvA, 100 % of F2 was in complex II, but even at 250 nM, RuvA2_Kap only bound 40 % of F2 as a mixture of complex I and II (data not shown). These results confirm that stable binding of RuvA to the fork requires interactions between the two tetramers. RuvA2_Kap complex was significantly defective in processing the fork (Fig. 6C). Over 50 % of the synthetic fork F2 was dissociated in reactions containing 250 nM RuvA and 300 nM RuvB, while less than 10 % of the substrate dissociated in parallel reactions using RuvA2_Kap (Fig. 6D). The majority of the F2 dissociation products represent processing in the direction opposite to fork reversal, as reported previously (25). These results show that the pronounced defect in the ability of RuvA2_Kap to bind to the synthetic fork (F2) resulted in a significant defect in processing.

Processing of Holliday junctions by RuvA2_Kap in vivo — As shown above, the mutant RuvA2_Kap was defective in forming stable complex II on model Holliday junctions and replication forks. As a consequence, the mutant was inefficient in branch migration of Holliday junctions and replication forks in vitro. It is important to correlate the observed defects of the RuvA2_Kap mutant with the currently known biological roles of RuvAB in vivo, namely processing of Holliday junctions and RuvAB-dependent reversal of stalled replication forks.

We first tested the ability of RuvA2_Kap to resolve HJ formed in vivo, by homologous recombination between intact DNA molecules. The *E. coli* strain JJC3207 (ΔruvA100::cat ΔrecG263::kan) (29) has a defect in homologous recombination which causes deficiency in Hfr conjugation. To test if RuvA2_Kap can process HJs in vivo, the ability of RuvA2_Kap to rescue the mutant phenotype of this strain was tested and compared to RuvA. The *ruvA2_Kap* coding sequence was cloned into the low copy plasmid pGB2 or in combination with the *ruvB* coding sequence to produce pGB-*ruvA2_Kap* and pGB-*ruvA2_KapB*. The genes were expressed under the control of the native *ruvAB* promoter. The plasmids pGB2, pGB-*ruvA*, pGB-*ruvA2_Kap* or pGB-*ruvA2_KapB* were transformed into the recipient, JJC3207. As the pGB-*ruvA2_Kap* plasmid codes for both RuvA2_Kap and RuvB, both proteins are expressed at the same levels when introduced into *E. coli*. This reduces any effects of altering the balance between RuvA2_Kap and RuvB, when RuvA2_Kap is over-expressed from the pGB2 plasmid while RuvB is expressed at low levels from the chromosome. The cells were grown to log phase then mixed with a His^+^ Hfr donor, plated on chloramphenicol minimal medium devoid of histidine and incubated for 48 hours. If successful homologous recombination had occurred, JJC3207 cells could grow on the plates lacking histidine as the His^+^ gene had been acquired from the Hfr donor via conjugation. Ratios of His^+^ versus total recipient colony forming units (cfu) are shown (Fig. 7A). Strain JJC3207 carrying vector pGB2 showed very low conjugation levels, less than 10^{−5} conjugants per cfu. Transformation of JJC3207 with pGB-RuvA resulted in much higher levels of conjugation, approximately 10^{−3} conjugants per cfu. When the cells were transformed with pGBruvA2_Kap or pGBruvA2_KapB the conjugation level was restored to nearly 10^{−3} conjugants per cfu, which is comparable to the rescued phenotype demonstrated by pGB-*ruvA*. Thus RuvA2_Kap is able to process HJs during conjugation as efficiently as wild type RuvA expressed from an exogenous plasmid. The complementation of the homologous recombination defect of JJC3207 by RuvA2_Kap was the same when expressed alone or in combination with RuvB on the pGB2 plasmid. This indicates that expressing extra copies of RuvB was not required for RuvA2_Kap mediated rescue of the JJC3207 conjugational defect.

The ability of RuvA2_Kap to process HJs in vivo was additionally tested during DNA single-strand gap and double-strand break repair, by assessing whether the mutant RuvA2_Kap was able to suppress the UV or Mitomycin C (MMC) sensitivity of a mutant strain, JIC2971 (ΔruvA100::cat) (29). JIC2971 was transformed with pGB-*ruvA*, pGB-*ruvAB*, pGB-*ruvA2_Kap* and pGB-*ruvA2_KapB* and the cells were exposed to increasing doses of UV light. JIC2971 cells transformed with pGB2 alone resulted in survival ratio of 10^{−1} cells at a UV dose of 40 J/m² (Fig. 7B). In contrast, the survival of JIC2971 transformed with pGB-*ruvA* was 100% at 40 J/m² UV dose, indicating that the cells were able to repair DNA lesions. JIC2971 cells transformed with either pGB-*ruvA2_Kap* or pGB-*ruvA2_KapB* gave survival profiles comparable to pGB-*ruvA* transformation at all doses tested,
indicating that RuvA2\textsubscript{KaP} is fully capable of resolving HJ made during the recombinational repair of UV lesions. The co-expression of RuvA2\textsubscript{KaP} with RuvB in JJC2971 cells very slightly improved survival which suggests that there may be a minor defect in the RuvA2\textsubscript{KaP} interaction with RuvB. In an additional assay, the JJC2971 cells were treated with 2 µg.ml\(^{-1}\) of MMC for 90 minutes to induce double-strand breaks in the DNA. These cells were then plated on LB spectinomycin overnight and the ratios of colony forming units in treated and untreated cultures were calculated (Fig. 7C). Introduction of pGB2 only resulted in a survival ratio of 10\(^{-5}\) cells. Introduction of pGB-ruv\textsubscript{A}, pGB-ruvA2\textsubscript{KaP} or pGB-ruvA2\textsubscript{KaP}B all resulted in a survival ratio of 10\(^{-2}\) cells indicating that the RuvA mutants were able to restore the cells resistance to MMC to the levels observed after introducing pGB-ruv\textsubscript{A}. These data suggest that RuvA2\textsubscript{KaP} is able to process Holliday junctions formed during recombinational-mediated repair of DNA lesions as efficiently as wild type RuvA.

**Stability of RuvA2\textsubscript{KaP} on Holliday junctions in vivo** — The defect of RuvA2\textsubscript{KaP} in maintaining stable binding to both sides of junction DNA as a double tetramer was demonstrated in vitro by its inability to inhibit cleavage of the junction by RuvC. We decided to examine this finding in vivo. In *E. coli*, the *rusA* gene encodes a Holliday junction resolvase carried on a cryptic prophage, but this gene in not normally expressed (8,29). The *E. coli* strain JJC2761 (*ArusABC rus-1*) does not encode RuvABC but the *rus-1* mutation results in *rusA* expression and the cells survive UV damage as RusA resolves Holliday junctions formed by recombinational-mediated repair (24,29). We used the JJC2761 strain as a tool to test the effect of RuvA2\textsubscript{KaP} on Holliday junction resolution by RusA in vivo. If a *rusA*-carrying plasmid is added to the strain, RuvA binding occludes the Holliday junction and prevents the action of RusA. Holliday junctions cannot be resolved, resulting in UV sensitivity and cell lethality. JJC2761 cells were transformed with pGB2, pGB-ruv\textsubscript{A}, pGBruvA2\textsubscript{KaP} or pGBruvA2\textsubscript{KaP}B and the survival of plasmid-harbouring cells subjected to different doses of UV-irradiation is shown in Fig. 7D. A similar level of protection against RusA-catalyzed HJ-resolution, resulting in UV sensitivity, was observed for pGB-ruv\textsubscript{A}, pGBruvA2\textsubscript{KaP} or pGBruvA2\textsubscript{KaP}B at 40 J/m\(^2\). At higher doses, the survival ratio of JJC2761 cells with pGBruvA2\textsubscript{KaP} or pGBruvA2\textsubscript{KaP}B was intermediate between those observed with the vector pGB2 and with the control plasmid pGB-ruv\textsubscript{A}, approximately 15-20 fold more cells survived compared to cells carrying pGB-ruv\textsubscript{A} at 100 J/m\(^2\). These data demonstrate that RuvA2\textsubscript{KaP} alone or with RuvB, could protect the Holliday junction from cleavage by RusA, but not as efficiently as wild type RuvA. The RuvAz3 and RuvAz87 mutants, which were deficient in inhibiting RuvC-mediated cleavage of HJs (Fig. 2B) were also defective in preventing RusA cleaving Holliday junctions in the JJC2761 strain (29).

*RuvA2\textsubscript{KaP} is deficient for replication fork reversal in vivo* — Finally, the ability of the mutant RuvA2\textsubscript{KaP} to reverse stalled replication forks in conjunction with RuvB was tested in vivo using the strain JJC3723 (*dnaEts recB ruvA100*). In cells carrying the temperature sensitive replication mutant *dnaEts*, RuvA is required for replication fork reversal (RFR) (24). In the strain JJC3723, RFR can be measured by re-introducing RuvA on a plasmid and measuring the linearised DNA formed in this *recB* mutant by RuvC cleavage of reversed forks (29).

JJC3723 cells transformed with pGB2, pGB-ruv\textsubscript{A}, pGB-ruvA2\textsubscript{KaP} and pGB-ruvA2\textsubscript{KaP}B constructs were grown at 30°C and then shifted to 42°C for 3 hours. The amount of linearised DNA in the cells was analysed by PFGE (pulse field gel electrophoresis) and quantified (Fig. 7E). Cells carrying the wild type RuvA plasmid, pGB-ruv\textsubscript{A}, resulted in 55 % linearization of DNA compared to 11 % DNA linearization in control cells with empty vector pGB2 and with the control plasmid pGB2, pGB-ruv\textsubscript{A} and pGB-ruvA2\textsubscript{KaP}B, the amount of DSBs increased from 11% to ~28%, but was still significantly lower than the levels of 55 % observed with pGB-ruv\textsubscript{A}, and than the ~70% breakage conferred by pGB-ruv\textsubscript{A}B in a similar strain (33). These data indicate that RuvA2\textsubscript{KaP}, which binds forks but is deficient in complex II formation in vitro, is unable to reverse forks in vivo. Thus, a single RuvA tetramer which cannot interact with a second RuvA tetramer on DNA is not sufficient to reverse forks in concert with RuvB. Since RuvA2\textsubscript{KaP} is proficient in processing Holliday
diagrams of two different branched substrates in vivo - Holliday junctions and stalled replication forks. To this end we used a defined RuvA mutant, RuvA2 KaP, designed as “tetramer-only” by replacing two amino acid residues (E127R, K119A) engaged in ionic pairs that stabilise interactions between two RuvA tetramers on the Holliday junction (supplementary Fig. 1). Several lines of evidence argue that the two substitutions in RuvA2 KaP do not affect the quaternary structure of the protein. The size exclusion data indicated that RuvA2 KaP forms the correct complexes in the correct amounts in solution (supplementary Fig. 2A). The protein is stable compared to wild-type RuvA when analysed on SDS PAGE (no degradation products were detected). Furthermore, like RuvA, RuvA2 KaP remains a tetramer when run in SDS, indicating that interactions within the tetramer are stable (supplementary Fig. 2A). Complex formation with RuvB analysed by SEC (supplementary Fig. 2C) indicates no defect in RuvA2 KaP-RuvB protein interaction, and finally RuvA2 KaP-HJ binding affinity is as wild-type (Fig. 1B). Therefore we consider it unlikely that the two substitutions in RuvA2 KaP affect the protein structure beyond destabilizing the double-tetramer. As expected, RuvA2 KaP was found to bind predominantly to HJs as complex I.

Tetramer only mutants are impaired in vitro for the formation of a RuvA-RuvB-HJ tripartite complex, for the stimulation of RuvB ATPase and branch migration activities; nevertheless, they promote homologous recombination in vivo. — We observed that in Mg2+ buffer the affinity of RuvA2 KaP for DNA remains similar to that of the wild-type protein but binding of the second tetramer is affected. This result suggests that Mg2+ has a greater effect on tetramer-tetramer interactions compared with modulating RuvA’s affinity for DNA. Interestingly, this result also reveals that complex II devoid of tetramer-tetramer interactions might form if the affinity for DNA of each of the two tetramers is high enough – albeit less stably than wild type. It supports the idea of RuvA co-existing on a HJ with RuvC (34). In fact, proteins are able to bind the opposite faces of a HJ without protein-protein interactions, as simultaneous binding to HJ was observed using two unrelated proteins, RuvA and the yeast mitochondrial protein YDC2 (35). The formation of unstable complex II devoid of tetramer-tetramer interactions correlates with a reduced ability of RuvA2 KaP to stimulate RuvB ATPase activity in vitro, and a defect in tripartite complex formation. Even at concentrations which allowed RuvA2 KaP complex II formation on HJs in Mg2+ buffer, RuvA2 KaP could not stimulate RuvB ATPase activity to wild-type levels. The loss of tetramer-tetramer interaction could lead to a deficiency in functional interactions with RuvB on DNA. SEC analysis confirmed that physical interactions between RuvA2 KaP and RuvB in the absence of DNA were not compromised, suggesting that RuvA2 KaP-RuvB interactions were not responsible for the reduced ATPase activity observed by RuvA2 KaP. However, formation of the tripartite complex was clearly affected. Additionally, RuvA2 KaP was defective in forming a RuvB complex when cross-linked to DNA, suggesting that stable formation of complex II via tetramer-tetramer interactions is critical for stabilizing RuvB on DNA. The decreased ATPase activity of a RuvA2 KaP mutant complex could reflect a role of tetramer-tetramer interaction in RuvA-facilitated communication required for two RuvB hexamers to function in concert. However, this is unlikely since RuvA2 KaP is deficient at supporting a single RuvB hexamer during branch migration of the four-way junction HJY3-hm in vitro, compared to a double tetramer of RuvA. This cannot be due to a requirement for communication between two RuvB hexamers as there is only a single RuvB hexamer, although it remains possible that communications between subunits of the hexamer are affected. It is most likely that the ATPase and branch migration deficiency of RuvB in the presence of RuvA2 KaP is due to an inherent inability of a RuvA single tetramer, or of a RuvA double tetramer devoid of tetramer-tetramer interactions, to stably tether RuvB to DNA. Alternatively, the wild-type double tetramer stabilized by tetramer-tetramer interactions may have an unknown mechanistic function required for stable RuvB branch migration, such as the separation of the strands.
by the central negative pin and channelling through the cruciform tunnels.

The in vitro defects of RuvA2Kap, namely inefficient complex II formation and poor branch migration in vitro, do not translate into any detectable recombination deficiency in vivo. In vivo studies indicated that RuvA2Kap supports RuvB mediated processing of Holliday junctions during conjugation and recombinational repair of UV and MMC induced DNA damage. Therefore, tetramer-tetramer interactions within complex II are not required for the formation of recombinant molecules, although it is possible that branch-migration tracks are shorter in the presence of RuvA2Kap compared to wild-type RuvA. Similarly, a surprising lack of correlation between in vivo homologous recombination proficiency and in vitro defects was previously observed for other separation-of-function mutants (RuvAz3 and RuvAz87, (29)). This paradox is confirmed here, where although they are recombination proficient in vivo, these mutant proteins show a defect in protection against RuvC-mediated resolution and in stimulation of RuvB-ATPase activity similar to that of RuvA2Kap. The recombination efficiency of mutant proteins that are less active than wild-type in several in vitro assays is an interesting observation that is not yet understood. The efficient RuvA2KapB Holliday junction processing in vivo may be due to cellular factors stabilising the complex on DNA or alternatively, cellular factors may exist that recruit RuvA to the Holliday junctions thus increasing RuvA2Kap localised concentrations. Such a cellular factor could be RecA which creates the HJ by strand invasion. Alternatively, because pGB2 is present as 10 copies per cell, it is possible that the extra RuvA2Kap molecules expressed from this plasmid are able to overcome the inherent defect of RuvA2Kap in forming complex II on HJs. Indeed, at high concentrations of RuvA2Kap in magnesium buffer, complex II was formed on HJs, allowing more efficient branch migration in vitro. A final possibility is that the RuvAB branch migration motor is overly efficient at processing HJs in vivo and that the ineffective branch migration of RuvA2KapB is still above the threshold of activity needed for HJ processing during homologous recombination in vivo.

The tetramer-only mutant is a separation-of-function mutant. Several ruvA and ruvB separation-of-function mutants were isolated based on in vivo genetic screening (29,33) and two were characterized in vitro (ruvAz3 and ruvAz87 (29); this work). These RuvAz mutants were previously shown to be defective for complex II formation in vitro and for replication fork reversal in vivo, suggesting a role for tetramer-tetramer interaction in replication fork reversal (29). However, these mutants were defective for several in vitro activities: complex II formation and DNA binding (particularly in the presence of divalent cations) and it was not possible to identify a RuvA function that was specifically required for RFR. RuvA2Kap allowed us to directly test whether one or two RuvA tetramers bind replication forks to support a single RuvB hexamer resulting in unidirectional fork processing during fork reversal. RuvA2Kap bound to synthetic replication forks with a lower affinity than wild type, contrary to the binding affinity of RuvA2Kap for HJs, which was as wild type. These data imply that RuvA2Kap binding to a synthetic fork is inherently unstable and that wild type RuvA tetramer-tetramer interactions are required for fork binding. When Mg2+ is included in the fork binding reaction, the affinity of RuvA2Kap is more defective compared to wild type than in the absence of Mg2+ and little RuvA2Kap complex I is observed. These data indicate that the formation of complex II on a synthetic replication fork has a stronger requirement for stable tetramer-tetramer interactions than formation of complex II on a HJ. This higher requirement of stable complex II formation on synthetic replication forks may be due to the nature of the substrate, as a RuvA tetramer binds DNA on its concave basic face which contains four grooves in a cross arrangement (14). Thus a HJ bound to the grooves would make more DNA protein contacts than a three-armed substrate. However, RuvA2Kap and wild type RuvA bind Y-junctions with a similar affinity (data not shown). The Y-junction and fork used in this study are identical in DNA sequence with the only difference between them being a single nick between the sister duplex arms of the fork substrate. Thus, it is likely that the structural differences between the rigid Y-junction and more flexible fork substrate accounts for the different interactions between these substrates and RuvA2Kap. This is confirmed by in vitro evidence that RuvA2Kap is defective at processing the fork substrate in comparison to RuvA and defective at fork reversal in a dnaEts mutant in vivo.
Like RuvAz3 and RuvAz87, RuvA2KaP is a separation-of-function mutant: able to branch migrate Holliday junctions but not to reverse forks. Furthermore, these data are contrary to the model of a single RuvA tetramer recruiting a single RuvB hexamer onto the parental duplex arms of a stalled fork and the subsequent processing in the direction required for fork reversal. Our data indicate that the formation of a RuvA complex II stabilized by tetramer-tetramer interactions is crucial for fork reversal in vivo. As discussed previously (29), this study supports the idea that RFR mediated by RuvAB is a process which requires the formation of an inherently unstable RuvAB complex (consisting of a single hexamer of RuvB) on a three-way junction which is less stable than a RuvAB complex (consisting of two RuvB hexamers) bound to a HJ. The requirement for stabilising this complex on a three-way fork junction is a double tetramer of RuvA with wild-type tetramer-tetramer interactions, whereas branch migration of a HJ can be carried out by an unstable double tetramer with two RuvB hexamers providing additional stabilisation through their contacts with RuvA.

Separation-of-function phenotype might result from various RuvAB defects, since different separation-of-function mutants were isolated in the ruvA as in the ruvB gene (29,33). Studies of ruvB mutants showed that mutations that presumably affect the ATPase activity of RuvB also confer a separation-of-function phenotype (33). The present study identifies the tight binding of two RuvA tetramers via tetramer-tetramer interactions as a property crucial for replication fork reversal but not for homologous recombination.

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FIGURE LEGENDS

Fig. 1. RuvA2KaP binding synthetic Holliday junction. (A) A representative EMSA is shown. Increasing concentrations of RuvA or RuvA2KaP were incubated with IRD700-labelled junction X12 in EDTA buffer and analysed by native 4 % PAGE. The expected positions of unbound HJ (crucifix schematic), complex I and complex II are indicated. DNA was visualised by the Odyssey LI-COR fluoro imaging system. (B) The fluorescence scans of the resulting bands from 6 independent EMSAs were quantified and the data plotted as a graph of the percentage of X12 molecules bound by RuvA or RuvA2KaP as a function of protein concentration. The error bars are SD. (C) A representative EMSA showing binding reactions with increasing concentrations of RuvA or RuvA2KaP incubated with IRD700-labelled X12 in Mg2+ buffer. As in (A), the expected positions of unbound HJ, complex I and complex II are indicated. The Protein-DNA complexes were analysed by native 4 % PAGE in TBM buffer. (D) 5 independent EMSAs of RuvA2KaP or RuvA with X12 in Mg2+ were quantified and the data plotted as a graph showing the percentage of X12 substrate bound by RuvA or RuvA2KaP as a function of protein concentration. The error bars are SD.

Fig. 2. Stability of complex II of RuvA mutants on Holliday junctions. (A) Increasing concentrations of RuvA mutants were incubated with X12, 100 nM RuvC was added and the reaction was incubated for 15 minutes at 37ºC. The cleavage products were analysed on native 4 % PAGE and a representative gel is shown. The positions of uncleaved HJ and cleavage product are indicated as schematics on the left. (B) 4 independent experiments were quantified and the data used to plot a graph of the percentage of X12 substrate as a function of RuvA/RuvA mutant protein concentration. The error bars represent SDs.

Fig. 3. ATP hydrolysis of RuvA mutant-RuvB complexes on synthetic HJs. (A) Time course of ATP hydrolysis. 100 nM of RuvA or RuvA2KaP were incubated with 500 nM RuvB and X12 at 37ºC. ATP hydrolysis is proportional to the release of inorganic phosphate (in µmols) as quantified
using a colourimetric assay. Three separate data sets were quantified and the data was plotted as the mol of ATP hydrolysed per mol of RuvB as a function of time. The error bars represent SD. (B) ATPase activity at two different concentrations of RuvA or RuvA2Kap incubated with 500 nM RuvB and X12 for 30 minutes. The experiment and graph were produced as described in (A). (C) RuvA, RuvA2Kap, RuvA2Z or RuvAz87 were either incubated alone or mixed with 500 nM RuvB. These proteins were then incubated with ATP for 10 min at 37°C in the presence of 200 ng μl−1 DX174 virion DNA. ATP hydrolysis was indirectly measured using a colourimetric assay to detect the amount of P\textsubscript{i} released. The data was used to plot a bar chart of mol of ATP hydrolysed per minute. The error bars represent SDs.

**Fig. 4. Interaction of RuvA2Kap with RuvB on HJ.** Increasing amounts of RuvA or RuvA2Kap were mixed with X12 in the presence of 300 nM or 600 nM RuvB in triethanolamine buffer containing ATP\textgamma{}S. The Protein-DNA complexes were cross-linked using 0.2 % glutaraldehyde and DNA-protein complexes were analysed by native 6 % PAGE. The positions of unprocessed HJ is shown schematically on the left.

**Fig. 5. Holliday junction processing of RuvA2Kap with RuvB.** (A) Branch migration of X12. Increasing concentrations of RuvA and RuvA2Kap were incubated with 5 ng of IRD700-labelled X12 and 250 nM RuvB at 37°C for 30 minutes. The reactions were stopped and analysed by 6 % native PAGE. A representative gel is shown, the positions of unprocessed HJ is shown schematically on the left. (B) Three independent X12-HJ branch migration experiments were quantified and the data was used to generate a graph showing the percentage of branch migration product formed as a function of protein concentration. The error bars represent SDs. (C) Branch migration of HJY3-hm. HJY3-hm was incubated with increasing concentrations of RuvA or RuvA2Kap and 250 nM RuvB at 37°C for 30 minutes. The data was used to generate a graph showing the percentage of branch migration product formed as a function of protein concentration. The error bars represent SD.

**Fig. 6. RuvA2Kap binding to a synthetic fork in EDTA.** (A) Increasing concentrations of RuvA or RuvA2Kap were incubated with fluorescently labelled F2 for 5 minutes on ice. Complexes of DNA with RuvA or RuvA2Kap were analysed by native 4 % PAGE. A schematic representation of the DNA substrate is shown, with the fluorescent labelled oligonucleotide represented by an asterisk (*). (B) 6 independent EMSAs were quantified and the data was used to generate a graph showing the percentage of F2 substrate bound as a function of protein concentration. The error bars represent SDs. (C) Increasing concentrations of RuvA2Kap or RuvA were incubated with a synthetic model fork (F2) and 300 nM RuvB. The reactions were incubated for 30 minutes at 37°C and analysed by native 6 % PAGE. Schematic representations of the substrate and the two possible branch migration products are indicated on the left. The first two lanes contain the two possible branch migration products as markers. (D) 6 independent fork branch migration experiments were quantified and the data was used to generate a graph showing the percentage of product formed as a function of protein concentration. The error bars represent SDs.

**Fig. 7. RuvA2Kap complex II binding stability and RuvA2KapB HJ and fork processing in vivo.** For figures A, C and E, error bars represent SD. For Figures B and D, error bars indicate the minimum and maximum values. (A) RuvA2Kap was tested for rescue of conjugation ability of the E. coli strain JC3207 (ruvA100 recG). RuvA2Kap was expressed from a pGB2 plasmid alone (pGB-ruvA2Kap) or in combination with RuvB (pGB-ruvA2KapB). The log\textsubscript{10} survival of conjugates/colony forming unit (cfu) was plotted as a bar graph. (B) The ability of RuvA2Kap to rescue UV sensitivity in a JC2971 (ruvA100::cat) strain was tested. JC291 was transformed with pGB2, pGB-ruvA, pGB-ruvA2Kap or pGB-ruvA2KapB. The ratio of colony forming units of treated versus untreated cells was calculated to derive log\textsubscript{10} survival ratio. (C) JC2971 cells were transformed with pGB2, pGB-ruvA, pGB-ruvA2Kap and pGB-ruvA2KapB and incubated with 2 μg ml\textsuperscript{−1} of mitomycin C for 90 minutes. The ratio of colony forming units of treated versus untreated cells was calculated to derive log\textsubscript{10} survival ratio. (D) RusA cleavage in vivo. JC2761 (ΔruvABC rus-1) E. coli cells were...
transformed with pGB2, pGB-ruvA, pGB-ruvA2kap or pGB-ruvA2kapB and exposed to UV radiation. The experiments were quantified and the data was used to generate a graph of the log₁₀ survival ratio of the transformants as a function of the dose of UV (J/m²). (E) Processing of synthetic forks by RuvA2kap in vivo. An E. coli strain, JJC3723 (dnapEts recBCts ruvA100) was transformed with pGB2, pGB-ruvA, pGB-ruvA2kap or pGB-ruvA2kapB. The cells were grown at 30°C and then shifted to 42°C for 3 hours, after which, fork reversal was assessed by the amount of double strand breaks generated, which was measured by the amount of linear chromosomal DNA entering a pulse field gel. The experiments were quantified and used to generate a histogram of the percentage of linear DNA for each strain.

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