RecA Dimers Serve as a Functional Unit for Assembly of Active Nucleoprotein Filaments†

Anthony L. Forget,‡,§ Michelle M. Kudron,‡,⊥ Dharia A. McGrew,|| Melissa A. Calmann,▽ Celia A. Schiffer, and Kendall L. Knight*

Department of Biochemistry and Molecular Pharmacology, Aaron Lazare Research Building, 364 Plantation Street, University of Massachusetts Medical School, Worcester, Massachusetts 01605

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ABSTRACT: All RecA-like recombinase enzymes catalyze DNA strand exchange as elongated filaments on DNA. Despite numerous biochemical and structural studies of RecA and the related Rad51 and RadA proteins, the unit oligomer(s) responsible for nucleoprotein filament assembly and coordinated filament activity remains undefined. We have created a RecA fused dimer protein and show that it maintains *in vivo* DNA repair and LexA co-protease activities, as well as *in vitro* ATPase and DNA strand exchange activities. Our results support the idea that dimeric RecA is an important functional unit both for assembly of nucleoprotein filaments and for their coordinated activity during the catalysis of homologous recombination.

The bacterial RecA protein is the founding member of a family of enzymes that catalyzes recombination between homologous DNA substrates, a family that includes the phage T4 UvsX, yeast Rad51, archael RadA, and human Rad51 proteins (*1*, *2*). To perform this function, these recombinase enzymes form a helical filament that assembles cooperatively on a single-stranded region of DNA resulting in the formation of an active nucleoprotein complex that subsequently interacts with a dsDNA substrate to catalyze a search for homology and DNA strand exchange (*1*, *3*, *4*). Early biochemical studies of RecA revealed that in the absence of substrate DNA the protein exists in solution as a complex heterogeneous mix of various oligomers (*5*−*8*), and later work showed that this mix included monomers, ring-shaped hexamers/heptamers, and short filaments ranging in length from 0.03 to 0.15 μm, as well as bundles of filaments (*9*−*12*).

The RecA nucleoprotein filament assembles in an ATP-dependent, highly cooperative manner (*13*−*16*). Kinetic analysis of nucleoprotein filament assembly has shown that RecA polymerization onto ssDNA can be divided into two major steps, (i) nucleation, which is rate limiting, and (ii) filament extension (*17*). However, the oligomeric heterogeneity of RecA in these studies precluded identification of a specific RecA oligomer, for example, monomers or dimers, as being an obligatory component of the assembly process, or whether various larger RecA oligomers, for example, trimers or hexamers, could participate in filament assembly. It was also found that RecA oligomers formed in the absence of ATP or ssDNA are not directly interconvertible with active nucleoprotein filaments and that disassembly of the preformed self-filaments was required prior to filament assembly on DNA (*18*, *19*). Again, however, the oligomeric state of the dissociated RecA could not be determined in these studies. Thus, the identity of the nucleating unit of recombinase filament assembly remains unclear.

In this study, we have spliced two copies of the *recA* gene into a single transcriptional unit and purified a version of the protein that we refer to as the RecA-FD[1] (fused dimer). RecA-FD shows wild-type RecA activities *in vivo* and catalyzes strand exchange *in vitro*. Our results show that formation of active RecA nucleoprotein filaments does not require monomeric RecA and suggest that oligomeric forms of the protein can participate in filament assembly.

EXPERIMENTAL PROCEDURES

Expression Constructs. The gene encoding wild-type RecA was expressed using a previously described construct, pTRecA420, in which *recA* is regulated by the tac promoter (*20*, *21*). The gene encoding the fused RecA dimer (*recA-FD*) was constructed as follows. Two point mutations, Lys6Ala and Arg28Ala, were introduced into the wild-type *recA* gene carried in plasmid pTRecA420 using the QuikChange protocol (Stratagene). This mutant *recA* gene was PCR amplified using a top strand primer that overlapped the *Nco*I restriction site (underlined) at the fMet initiation codon (5′-GG AGT GAT GCC ATG GCT ATC GAC G-3′)

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* Tel (508) 856-2405; fax (508) 856-6231; e-mail kendall.knight@umassmed.edu.

‡ A.L.F. and M.M.K. contributed equally to this work.

§ Current address: Section of Microbiology, University of California, Davis, 345 Briggs Hall, One Shields Avenue, Davis, CA 95616-8665.

⊥ Current address: Department of Genetics, Yale University, 333 Cedar Street, NSB 396, New Haven, CT 06520.

|| Current address: Life Sciences Graduate Program in Molecular and Cell Biology, Brandeis University, Mailstop 008, Waltham, MA 02454-9110.

▽ Current address: Global Biologics Supply Chain, 200 Great Valley Parkway, Mailstop M-7, Malvern, PA 19355.

[1] Abbreviations: RecA-FD, RecA fused dimer; PCR, polymerase chain reaction; UV, ultraviolet; EDTA, ethylenediamine tetra-acetic acid; Tris, tris(hydroxymethyl) aminomethane; DTT, dithiothreitol.

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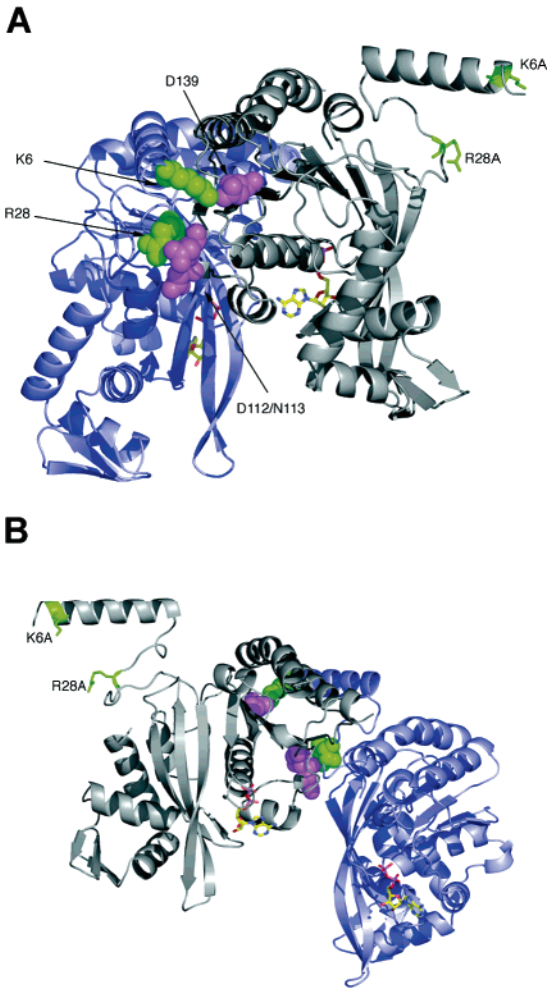

FIGURE 1: RecA dimer structure. These views show (A) the outside surface and (B) inside surface of a RecA dimer as it exists within the helical RecA protein filament. The α-carbon backbones of the two subunits are colored gray and blue, with side chains of residues Lys6 and Arg28 in green, Asn112, Asp113, and Asp139 in violet, and ADP in atom colors. The Lys6Ala and Arg28Ala in the N-terminal subunit of the fused dimer are indicated. The images were created using PyMOL (DeLano Scientific LLC) and the pdb file 1REA.

and a bottom strand primer that adds a five-residue linker (Gly_3Ser_2) at the RecA C-terminus and also contains an *Nco*I restriction site (underlined, 5′-GCA TGC CAT GGA GCT CCC GCC TCC AAA ATC TTC GTT AGT TTC TGC-3′). This PCR product was digested with *Nco*I and ligated into the *Nco*I site in pTRecA420, and candidates resulting in two fused *recA* genes carrying the Lys6Ala and Arg28Ala substitutions only in the N-terminal copy were screened by restriction mapping (see Figure 1). Identification of the correct construct was confirmed by DNA sequencing.

Recombinational DNA Repair and LexA Co-Protease Activities in Vivo. Recombinational DNA repair activity was measured following exposure of cells to varying doses of UV irradiation as previously described (*20*). LexA co-protease activity was measured using strain DE1663′ and the appearance of red vs white colonies on MacConkey lactose plates as previously described (*22*).

Protein Purification. Wild-type RecA was purified as previously described (*23*). RecA-FD was purified using a similar procedure with the following modifications. Cells were lysed by sequential additions of lysozyme (final concentration = 0.2 mg/mL), EDTA (final concentration = 0.5 mM), and Brij-35 (final of 0.5%). The suspension was stirred at 4 °C for 30 min after each addition followed by brief sonication, and the resulting lysate was centrifuged for 90 min (Sorval SA-600 at 13 500 rpm). The supernatant was recovered, and proteins were precipitated by addition of 0.32 g/mL $(NH_4)_2SO_4$ and incubation overnight at 4 °C. Precipitate was recovered by centrifugation, dissolved in R buffer (20 mM Tris, pH 7.5/10% glycerol/1 mM DTT/0.1 mM EDTA) and dialyzed extensively against the same buffer. Nucleic acids were removed by addition of streptomycin sulfate (final concentration = 0.5%) followed by centrifugation. The supernatant was loaded onto a 40 mL DEAE (DE52) column equilibrated in R buffer/50 mM NH_4Cl, and proteins were eluted using a 50−400 mM NH_4Cl gradient. Fractions containing RecA-FD were pooled and precipitated as above; protein was dissolved, dialyzed using R buffer/25 mM NaCl and loaded onto a ssDNA cellulose column (8 mL) equilibrated in the same buffer. The column was washed with two column volumes of R buffer/25 mM NaCl, and RecA-FD was eluted using one column volume of this buffer containing 2 mM ATP. Fractions containing RecA-FD were pooled, precipitated as above, dissolved in R buffer, and dialyzed extensively against the same. The concentration was determined spectrophotometrically (ϵ_{280} = 0.59 mg^{-1}·mL) and by comparison to standards on SDS gels. Glycerol was added to a final concentration of 25%, and samples were quick frozen in liquid nitrogen and stored at −80 °C. Both the wild-type RecA and RecA-FD proteins were judged to be >95% pure by Coomassie-stained SDS polyacrylamide gels. In all RecA-FD preparations, approximately 2−5% of the protein appeared as monomeric RecA (Figure 2). Because protease inhibitors were added to the cells immediately before freezing and were maintained during the early steps of purification, this likely resulted from intracellular proteolysis near the Gly_3Ser_2 linker. This contaminating monomeric RecA, which consists of equal amounts of wild-type RecA and the Lys6Ala/Arg28Ala mutant, can increase the measured activities of the fused dimer preparation by no more than 5%. However, in no case did any of the activities measured *in vivo* or *in vitro* show a decrease approaching 20-fold (see Results). Therefore, the fused dimer itself maintains significant catalytic activity. For all results, the

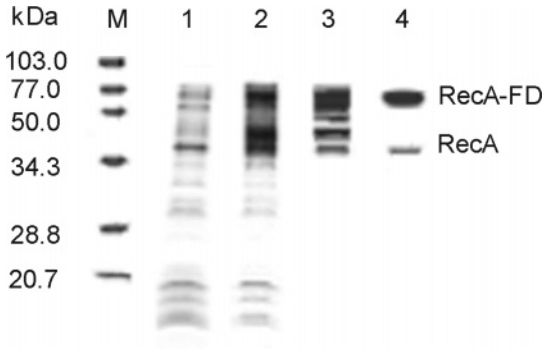

FIGURE 2: Purification of the RecA-fused dimer protein. SDS−polyacrylamide gel (11%) showing molecular mass markers (lane M), crude extract supernatant before and after induction with IPTG (lanes 1 and 2, respectively), pooled fractions from DE52 column gradient (lane 3), and elution from ssDNA cellulose (lane 4). The final step produced RecA-FD protein at >95% purity with less than 5% contamination of monomeric RecA resulting from intracellular proteolysis (see Experimental Procedures).

concentration of both wild-type RecA and the fused dimer refers to monomeric protein.

ATPase Activity. Hydrolysis of [α-^{32}P]-dATP was performed essentially as described (*24*). Reactions included the following components: 20 mM Tris (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.0 mM DTT, 0.5 mM [α-^{32}P]-dATP (20 μCi/mL), 2.0 μM RecA or RecA-FD protein, and 25 μM ss-φX174 DNA (concentration in bases). Percent hydrolysis as a function of time over 40 min was measured by scanning polyethyleneimine chromatography plates using a Molecular Imager FX and QuantityOne software (Bio-Rad).

Gel Shift DNA Binding Assay. Reactions (10 μL) contained 20 mM triethanolamine-HCl (pH 7.5,) 1 mM DTT, 10 mM $MgCl_2$, 0.1 mg/mL BSA, 100 nM 5′-fluorescein-labeled 95-base oligonucleotide (concentration in bases), and the indicated amounts of protein. The oligonucleotide was made using an ABI392 synthesizer, and fluorescein was conjugated in the last step of the synthesis using 5′-fluorescein phosphoramidite (Glen Research). The sequence is as follows: 5′-AGA CGA TAG CGA AGGCGT AGC AGA AAC TAA CGA AGA TTT TGG CGG TGG TCT GAA CGA CAT CTT TGA GGC GCA GAA AAT CGA GTG GCA CTA ATA AG-3′. Reactions were incubated at 37 °C for 30 min followed by addition of 2 μL of loading buffer and were loaded onto a 1.0% agarose gel and electrophoresed at 100 mV in 0.5 × TBE buffer with 10 mM $MgCl_2$. DNA was visualized by excitation at 473 nm using a Fuji Film FLA-5000 multifunctional imaging system (Fuji) and Image Reader software (Fuji).

DNA Strand Exchange. Reaction mixtures contained 25 mM Tris Acetate (pH 7.5), 10 mM MgOAc, 5% glycerol, 1 mM DTT, 8 mM phosphocreatine, 10 units/mL creatine kinase, single-stranded circular DNA (φX174, 20 μM bases), and 6.0 μM RecA or RecA-FD. Reaction mixtures were preincubated at 37 °C for 10 min, at which point linear duplex DNA substrate (*Xho*I digested φX174 virion DNA) was added to a final concentration of 26 μM (bases), and reaction mixtures were incubated an additional 10 min. Strand exchange was initiated by addition of a premixed solution containing 2 μM single-stranded DNA binding protein (SSB, US Biologicals) and 3 mM ATP. Samples (10 μL) were taken at indicated times, and strand transfer was terminated by addition of 2 μL of a stop buffer containing 5% SDS, 20% glycerol, 60 mM EDTA, and proteinase K to a concentration of 1 mg/mL. After incubation at 42 °C for 30 min, samples were analyzed by electrophoresis through a 0.8% agarose gel run in 40 mM Tris acetate and 2 mM EDTA. Gels were processed by staining in Vistra Green fluorescent stain (Amersham Pharmacia, Inc., 1:10 000) for 60 min and then analyzed by Image Reader 1 Laser/1 Image analysis at 473 nm on a phosphorimager (Fuji Films). The gels were quantified using Image Gauge software, v. 3.1.

Electron Microscopy. Samples were prepared for electron microscopy as previously described (*25*). Protein was 2.0 μM, ATPγS was 1.0 mM, and φX174 single-stranded circular DNA was 0.5 μM. Reactions with φX174 single-stranded circular DNA included 0.03 μM *Escherichia coli* SSB. Reactions were spread onto thin carbon films on holey grids (400 mesh), stained with 1% uranyl acetate, and visualized using a Philips CM10 electron microscope.

Table 1. DNA Repair, Co-Protease, and ATPase Activities for Wild-Type RecA and the RecA-FD Protein

protein	UV survival[a]	MMC survival[a]	LexA co-protease[a]	ATPase[b]
RecA	1.0	1.0	1.0	0.3 (−ssDNA) 18.3 (+ssDNA)
RecA-FD	1.0	0.9	1.0	0.3 (−ssDNA) 18.0 (+ssDNA)

[a] Fractional activity relative to wild-type RecA. [b] Units are mol of ADP/(min·mol of enzyme).

RESULTS

Design of the RecA Fused Dimer and in Vivo Activities. Our previous studies of two *recA* mutants, RecA K6A and RecA R28A, showed that each reduced formation of free protein filaments but formed nucleoproteins on ssDNA. Additionally, each purified mutant protein showed both a lower rate and a lower extent of DNA strand exchange relative to wild-type RecA over a 60 min time course (*21*). Therefore, we originally created the RecA fused dimer carrying the N-terminal K6A and R28A substitutions to investigate the possibility that this protein could be used in structural studies designed to reveal the filament subunit interface in the active form of RecA. While the subunit interface at the dimer junction is identical to wild-type RecA (Figure 1), we expected the N-terminal substitutions to prohibit further assembly of RecA filaments. We were surprised, however, to find that the *in vivo* activities of RecA-FD mimicked those of wild-type RecA. Expression of the plasmid-borne *recA-FD* gene (pTRecA-FD) in the Δ*recA* strains MV1190 or DE1663′ rescued the extreme sensitivity of each strain to mitomycin C and UV light to the same extent as expression of the wild-type *recA* gene (pTRecA420; Table 1). Additionally, analysis of the LexA co-protease function using a MacConkey lactose plate assay showed the RecA-FD protein to have the same level of DNA damage-inducible activity as wild-type RecA (Table 1). Thus, the ability of RecA-FD to catalyze LexA cleavage and recombinational DNA repair *in vivo* appears similar to that of wild-type RecA. Therefore, we exploited this protein to further investigate questions regarding the identity of the nucleating protein unit for recombinase filament assembly, as well as possible identification of a functional unit within an active filament.

Protein Purification. The RecA-FD protein was purified using procedures similar to those used for wild-type RecA (see Experimental Procedures). All RecA-FD preparations contained 2−5% monomeric RecA, despite the fact that protease inhibitors were present throughout cell harvesting and the purification procedure (Figure 2). However, as explained below (see Discussion), this small level of monomeric RecA, which likely arises from intracellular proteolysis in the region of the Gly_3Ser_2 linker, could in no way account for the *in vivo* or *in vitro* activities observed for the RecA-FD protein.

ATPase Activity. Purified wild-type RecA and the RecA-FD proteins showed similar levels of both basal and ssDNA-dependent ATPase activity (Table 1). Turnover numbers for both wild-type RecA and RecA-FD in the absence and presence of φX174 ssDNA were calculated as 0.3 and 18.3 mol of ADP·min^{-1}·mol^{-1} of RecA, respectively.

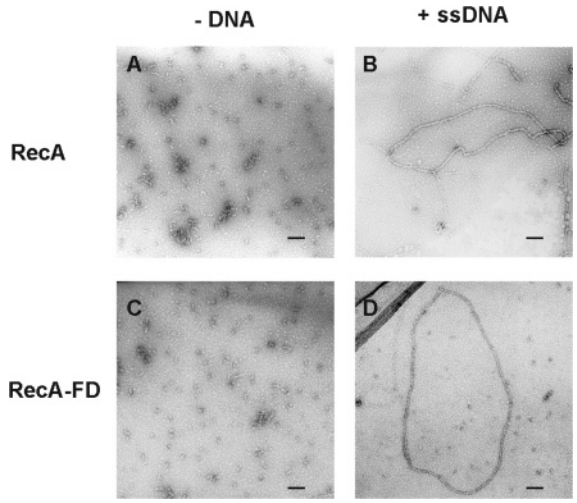

FIGURE 3: Electron micrographs of RecA and RecA-FD proteins with and without ssDNA. Wild-type RecA (A, B) and RecA-FD protein (C, D) were incubated as described in Experimental Procedures in the presence of ATPγS and the absence of ϕX174 phage ssDNA (A, C) or in the presence of both ATPγS and ϕX174 phage ssDNA (B, D). Black bar in each panel equals 0.1 μM.

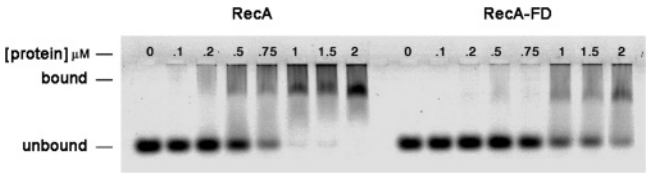

FIGURE 4: Gel shift DNA binding assay of RecA and RecA-FD proteins. Proteins were incubated at the indicated concentrations with a 5′ fluorescein-labeled 95-base oligonucleotide, and free and protein-bound DNAs were separated using agarose gel electrophoresis as described (see Experimental Procedures).

Nucleoprotein Filament Formation and DNA Binding. The ability of the RecA-FD protein to form an active nucleoprotein filament was assessed by electron microscopy and gel shift DNA binding assays. Electron micrographs of both wild-type RecA and RecA-FD in the absence of DNA show that both proteins form a mixed population of different sized oligomers (Figure 3, panels A and C). Similar results have been observed previously by several groups using either electron microscopy or various hydrodynamic methods (*9*−*12*). In the presence of ϕX174 ssDNA, both proteins form cooperatively assembled nucleoprotein filaments that show a striated helical pattern typical of RecA−DNA complexes (Figure 3, panels B and D). Despite the similar appearance of the nucleoprotein filaments, gel shift DNA binding assays reveal that the affinity of RecA-FD for a 95-base oligonucleotide is reduced compared with wild-type RecA. The gel in Figure 4 is representative of four different experiments run under similar conditions, and in each case, we estimated half-maximal DNA binding to be approximately 0.6 μM for wild-type RecA and 1.5 μM for RecA-FD. Therefore, despite RecA-FD being able to form wild-type-like nucleoprotein filaments, the ssDNA binding affinity of RecA-FD is reduced approximately 2.5-fold.

DNA Strand Exchange. DNA strand exchange assays were performed using single-stranded circular and homologous linearized duplex ϕX174 DNAs. When wild-type RecA is used, joint molecule intermediates are seen at 5 min, along with a small amount of the final reaction product, nicked circular DNA (Figure 5). The reaction goes to completion between approximately 30 and 45 min, in agreement with previous studies (*21*, *26*). By comparison, joint molecule intermediates are not observed until 15 min in reactions containing the RecA-FD protein, and discernible amounts of product appear at approximately 45 min. The reaction is still progressing at 90 min, at which time approximately 50% of the substrate duplex DNA has been converted to nicked circle products. Averages obtained from three separate experiments showed that the initial rate of strand exchange measured by substrate uptake was approximately 4-fold slower for the RecA-FD protein, whereas the rate of appearance of final product (nicked circles) was approximately 8-fold slower.

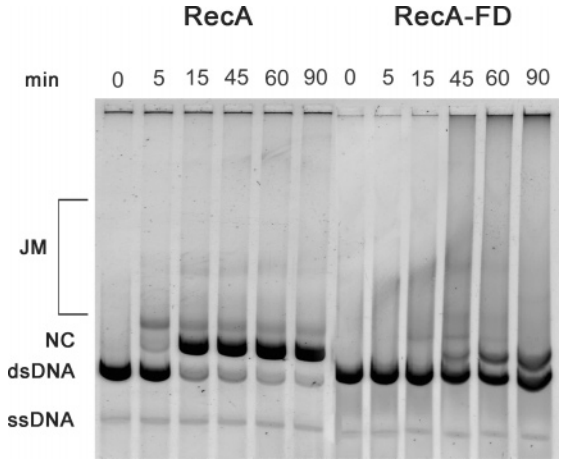

FIGURE 5: DNA strand exchange catalyzed by the RecA and RecA-FD proteins. Strand exchange reactions were performed as described (see Experimental Procedures) and included 20 μM ϕX174 ss- and dsDNAs (concentration in bases), 6.0 μM RecA or RecA-FD proteins, 2.0 μM SSB, and 3.0 mM ATP. Samples were removed at the indicated times, and substrates and products were separated by agarose gel electrophoresis. Products of the reaction include joint molecule intermediates (JM) and final product nicked circles (NC).

DISCUSSION

In this study, we show that a covalently fused dimer of the RecA protein is functional both *in vivo* and *in vitro* for activities associated with wild-type RecA, for example, repair of UV-mediated DNA damage, cleavage of the LexA repressor, DNA-dependent ATPase, and DNA strand exchange. Measurements of DNA repair and co-protease activities carried out *in vivo* show that the RecA-FD protein is as active as wild-type RecA. Biochemical assays show similar ATP turnover numbers for RecA-FD and wild-type RecA, while the ssDNA binding affinity is reduced approximately 2-fold for the RecA-FD protein. DNA strand exchange assays show that the activity of RecA-FD is approximately 5-fold slower than that of wild-type RecA, a result that may be in part attributed to the decrease in DNA binding affinity for the RecA-FD protein. We note that previous studies of the RecA K6A and RecA R28A mutant proteins show a decreased rate and extent of DNA strand exchange relative to wild-type RecA (*21*). Therefore, the decrease in strand exchange activity of the RecA-FD protein carrying both substitutions is most likely due to the mutations themselves rather than any effect of the dimer fusion. We also note that different preparations of RecA-FD protein typically have a low level of monomeric RecA, between 2%

and 5% of the total purified protein. Therefore, the observed activities clearly do not result from residual monomeric RecA; rather all activities measured reflect those catalyzed by the RecA-FD protein.

Many studies have shown that the oligomeric state of free RecA protein is exquisitely sensitive to a variety of solution conditions including protein concentration, ionic strength, and temperature and that at the protein concentrations typically used in DNA strand exchange assays, RecA exists as a complex mix of various oligomeric forms (*9*−*12*, *27*−*30*). The fact that the RecA-FD protein functions both *in vivo* and *in vitro* demonstrates that dimeric RecA can serve as the nucleating unit for filament assembly onto ssDNA. Biochemical and structural evidence supports the idea that inactive oligomers of RecA must disassemble prior to the assembly of active nucleoprotein filaments (*18*, *19*, *31*−*33*), and our data now suggest that disassembly does not necessarily need to progress all the way to monomeric RecA. At the RecA concentrations present in cells both before DNA damage and following DNA damage-mediated induction of *recA* expression, it is likely that most RecA exists in some oligomeric form. This is supported by analyses performed by Ruigrok and DiCapua (*28*) in which they estimated that aggregates of RecA could account for greater than 70% of the total RecA in the cell. In fact, recent studies now provide a direct demonstration that greater than 50% of cellular RecA exists in clusters not associated with DNA (*34*). While solution conditions have been manipulated to show that monomeric RecA can indeed initiate nucleoprotein filament formation (*35*), the ability of dimeric RecA to serve as a nucleating unit of filament assembly on ssDNA may be a cellular necessity given that, as described above, significant amounts of monomeric RecA are not likely to be present especially following induction of *recA* expression.

The idea that there is functional coupling between subunits in an active recombinase nucleoprotein filament has been well established by numerous studies (*17*, *36*). Cooperative binding of RecA and Rad51 to DNA demonstrates coordinated subunit interactions during nucleoprotein filament assembly (*13*, *15*, *16*). Earlier modeling of DNA binding data supported the idea that various oligomeric forms of RecA could interact directly with DNA (*37*), and subsequent biochemical studies suggested a subunit nonequivalence within an active nucleoprotein filament regarding both NTP and DNA binding (*38*). Additionally, recent studies describe a coordinated wave of ATP hydrolysis that propagates through a RecA filament that is likely linked to the enzyme's motor function responsible for catalysis of DNA strand exchange (*39*). Although the number of subunits that form an interacting unit within a recombinase could not be identified in any of these studies, there is intriguing structural evidence supporting the idea that recombinase dimers may contribute significantly to the underlying functional coordination within a recombinase filament. Electron microscopic analyses of *Thermus aquaticus* RecA in the absence of DNA revealed that a large population of hexameric rings was divided between those with 6-fold symmetry and those with 3-fold symmetry, with the latter observed to be trimers of dimers (*40*). While at that time it was not clear whether this 3-fold symmetry would be maintained to any degree within an active recombinase filament, new structural data strongly supports this idea. In the X-ray structure of yeast Rad51, Conway et al. (*41*) find that alternate subunit interfaces are different and suggest that a dimer may be the functional unit within the active recombinase filament.

Our results clearly demonstrate that RecA monomers are not an obligatory protein unit required for nucleoprotein filament assembly. Together with the published data discussed above, our data support a model in which dimeric RecA can serve as a nucleating unit for assembly of an active nucleoprotein filament and may also serve as a functional unit within the filament. Of course, this does not preclude monomers as also participating in these roles, and given the heterogeneous complexity of the RecA oligomeric population both *in vitro* and *in vivo*, it may be that more than one type of oligomer, for example, both monomers and dimers, can carry out both functions.

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